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14. ABSTRACT

This project ultimately aimed to identify the role of inhibin alpha (INHA) in advanced prostate cancer. The hypothesis tested was that INHA is tumor promoting and pro-metastatic in advanced prostate cancer (PCa).

At the end of this project, we have made significant progress towards understanding the role INHA in advanced prostate disease. We have demonstrated that increased INHA expression in highly aggressive, metastatic and androgen-independent PCa cell line, PC3, further promotes its tumor growth and metastatic ability. Increase in metastasis was further evident by increase lymph vessel density (LVD) and lymphatic invasion by the cancer cells. This was also accompanied by increase in VEGF-A and VEGF-C expression. *In vitro* tube formation assays has shown that Inhibin B and not Inhibin A induces significant lymphatic endothelial cell (LEC) tube formation. Furthermore, we conducted a cross-sectional study to determine a link between INHA expression and a number of clinicopathological parameters including Gleason score, surgical margin, extracapsular spread, lymph node status and VEGF-R3 expression which are well established prognostic factors of PCa. Elevated expression of INHA in primary PCa tissues showed a higher risk of PCa patients being positive for clinicopathological parameters outlined above. This study is the first to demonstrate a pro-tumorigenic and pro-metastatic role for INHA in the androgen-independent stage of metastatic prostate disease. Our results also suggest that INHA expression in the primary prostate tumor can be used as a predictive factor for prognosis of PCa.

Our work on understanding the mechanism suggests the involvement of ERK/MAPK signaling pathway through which INHA promotes tumor growth and metastasis. Change in this pathway was not evident in PCa cell line demonstrating tumor suppressive role of INHA. The outcomes of these experiments contribute significantly to our understanding of the role of INHA in the process of prostate carcinogenesis and open the way to explore the therapeutic potential of targeting the identified pathway in the treatment of PCa progression and metastasis.

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INTRODUCTION

This project ultimately aimed to identify the role of inhibin-α subunit (INHA) in prostate carcinogenesis. To date the role of INHA in reproductive cancers is equivocal. This project tested the hypothesis that increased expression of INHA in advanced prostate cancer (PCa) promotes tumor growth and the spread of cancer cells to the lymph nodes. We have made significant process towards proving the hypothesis and implicating INHA as being one of the factors inducing metastatic prostate disease. We have shown that increased INHA in PC3 PCa cell lines increases tumor growth and metastasis, which is associated with increase in VEGF-C expression in the cell line. Our results also suggest that Inhibin B and not Inhibin A is responsible for the tumor and metastasis promoting effect of inhibin in PCa. In addition, we have provided the biological mechanisms affected by increased INHA expression in prostate carcinogenesis. Using clinical specimens we have demonstrated a relationship between INHA expression and well established clinicopathological parameter of PCa. Detailed outcomes of the project are discussed below.

BODY

Task 1: To investigate the tumor promoting and pro-metastatic role of INHA using *in vitro* and *in vivo* models (Months 1-6).

a. Immunohistochemistry for human mitochondria on tissues (primary prostate tumors and lymph nodes (LNs)) harvested from study already completed will show the presence of human cells in the primary and secondary tumors.

We have completed the aims of Task 1a during the first six months of the project. Specifically, this involved using immunohistochemistry to show presence of human cells in the harvested tissues thereby validating our preliminarily observations; *in vivo* data from INHA over-expressing cells showed increased tumor size following orthotopic injection and a 3.5 fold increase (75% versus 20%) in the incidence of metastasis from the primary tumor to surrounding LNs compared to controls. Monoclonal human mitochondria antibody was used to determine the presence of human cells in the tumors (primary prostate tumors and LNs). We also used monoclonal R1 antibody to determine INHA expression in tumors. For detailed description of the methodology see Appendix 1.

Positive immunostaining for human mitochondrial protein confirmed that the primary and secondary tumors originated from intra-prostatic injection of human cells. INHA immunostaining was used to confirm INHA expression in tumors (Fig 1A & B; *left*). INHA over-expression in PC3 cells had no effect on orthotopic tumor take but a significant increase in the primary prostate tumor size ($p = 0.005$) was observed (Fig 1A; *middle & right*). INHA over-expression in PC3 significantly increased the incidence of lymph node tumors ($p = 0.0341$) and lymph node tumor size ($p = 0.0047$) compared to the empty vector (EV)-transfected clones (Fig 1B; *middle & right*).

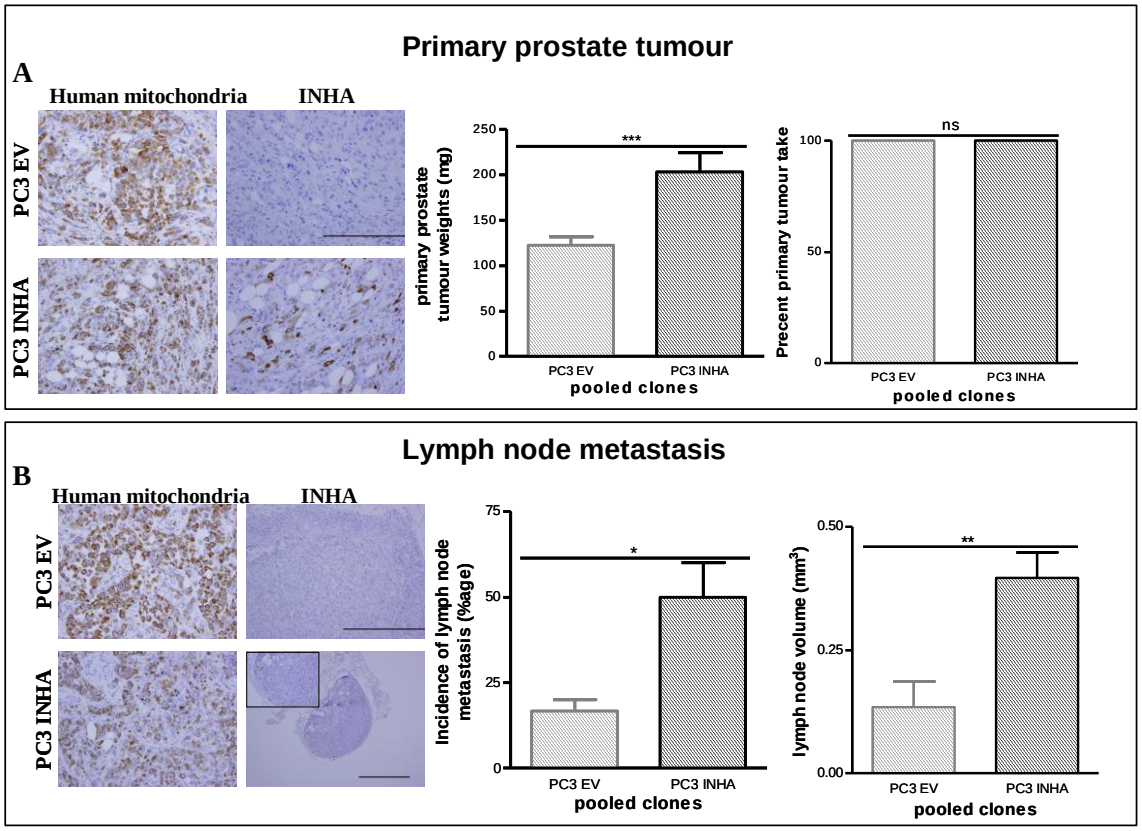

Fig 1 Effect of INHA over-expression on primary prostate tumor growth and lymph node metastasis. A-B; *left* Immunohistochemistry of primary prostate and lymph node tumors using human mitochondria and INHA staining confirmed the human origin of the cells in PC3 inoculated mice and INHA expression in the tumors. Bar 200 & 500μm. **A;** Primary prostate tumor weights *(middle)* and primary prostate tumor take *(right)*. **B;** Incidence of lymph node metastasis *(middle)* and lymph node volume *(right)*. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and no significant (ns) difference between the mean of the EV clones and the mean to the INHA-transfected clones. The bars represent: EV-transfected PC3 clones in grey, INHA-transfected PC3 clones in black. Data shown as mean ± SE of the mean.

b. Immunohistochemistry for human mitochondria and LYVE-1 on the primary prostate tumors will determine lymph vessel density (LVD) in the intratumoral, peritumoral and normal regions in the tissues.

We have completed the aims of Task 1a during the first six months of the project. Changes to LVD and lymphangiogenesis are often associated with metastatic spread of cancer cells to the LNs (1, 2). To understand the mechanisms and to provide proof of metastatic spread observed in the mice injected with INHA-positive cells we stained PC3 INHA and EV orthotopic tumors for LYVE-1, and human mitochondrial antibody to determine LVD and the degree of invasion of tumor cells into lymphatic vessels

(lymphatic invasion) in the tissues (Fig. 2a). Stereological analysis of these tumors revealed a significant increase ($p = 0.0023$) in the LVD in the intratumoral regions with no difference in LVD in peritumoral and surrounding non-malignant regions of INHA-positive tumors compared to the controls (Fig. 2b). Data also revealed significant increase in lymphatic invasion in the intratumoral ($p = 0.0002$), peritumoral ($p = 0.0225$) and non-malignant ($p = 0.0077$) regions of the tissue in INHA-positive tumors compared to the controls (Fig. 2c). For detailed description of the methodology see Appendix 1.

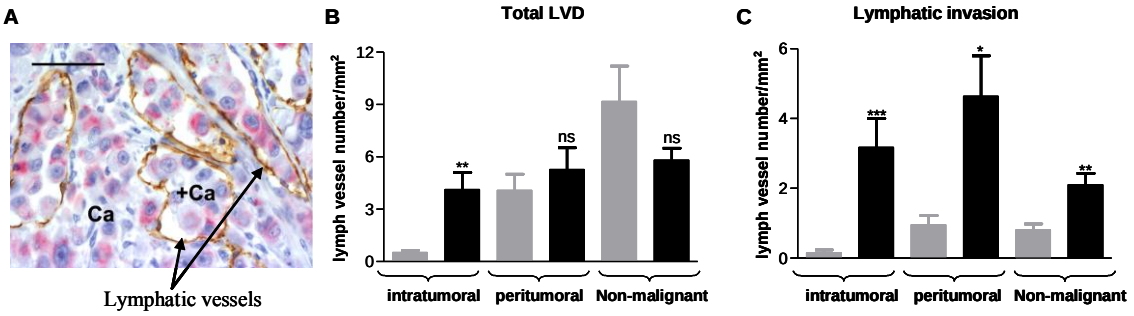

Fig 2 Increase in lymphatic vessel density and lymphatic invasion in PC3 tumors. *A,* Lymphatic vessels (LVs) were stained with LYVE-1 antibody (brown) and human prostate cells (Ca) with human mitochondria antibody (purple). Bar 50μm. The total number of LVs *(B)* and LVs with cancer cells in their lumen *(C)* (for example of such a vessel see "+Ca" in panel A) in the intratumoral, peritumoral and non-malignant (benign region adjacent to the tumor) regions of the primary prostate tumor were counted. * p 0.01 – 0.05, ** p 0.001 – 0.01, *** $p < 0.001$ and no significant (ns) difference between LVD in INHA over-expressing primary tumor compared to EV tumors. The bars represent: EV-transfected PC3 clones in grey, INHA-transfected clones in black. Data shown as mean ± standard error of the mean.

c. Collection of fresh prostate tissues from 3 prostate cancer patients, isolation of lymphatic endothelial cells (LECs) from and culturing them in the presence of PCa cells with and without INHA expression and/ or recombinant inhibin protein will determine the effect of the cancer cells and recombinant inhibin protein on LEC tube number and length.

We have completed the aims of Task 1c. Before this work begins, we were required to obtain Human Ethics approval to access human prostate tissues from patients undergoing radical prostatectomy surgery. Our laboratory already holds a human ethics approval at East Epworth Hospital, Boxhill, Melbourne, Australia to obtain fresh prostate tissues from patient undergoing surgery for another project "Role of tumor stroma in prostate carcinogenesis". The original application was amendment to include access of tissues for isolating human lymphatic endothelial cells. The primary approval was granted from Epworth Human Ethics committee (Approval Number: 34306 on 06 December 2006 [see appendix 2]. Secondary approval was granted from Monash University Standing Committee on Ethics in Research Involving Humans (Approval Number: 2004/145MC)

on 13 June 2007 [see appendix 3], which was necessary since some staff involved in the project are employees of Monash University.

We have successfully completed practice experiments of isolating LECs from prostate tissue. However, the isolated LECs proved difficult to use in our hands. It either differentiated in culture or did not give enough cells numbers to perform all the experiments. In order to successfully complete this aim, we collaborated with Dr Marc Achen and Dr Steven Stacker at the Ludwig Cancer Institute, Melbourne, Australia and were able to obtained LEC isolated from skin to do the proposed experiments.

The goal of this aim was to use LEC and culture them in the presence of PC3 PCa cells with and without INHA expression and/ or recombinant inhibin protein will determine the effect of the cancer cells and recombinant inhibin protein on LEC tube number and length. To determine if human recombinant Inhibin A and Inhibin B had any effect on LEC tube formation we seeded LEC in media with different dosages of the recombinant proteins or PBS vehicle control (only the results from the highest dose is presented here). The results showed a significant increase in LEC tubes formation (p=0.0087) and tube length (p=0.0012) in the presence of Inhibin B at a higher dose (20µg/ml) compared to Inhibin A at the same dose (Fig 3).

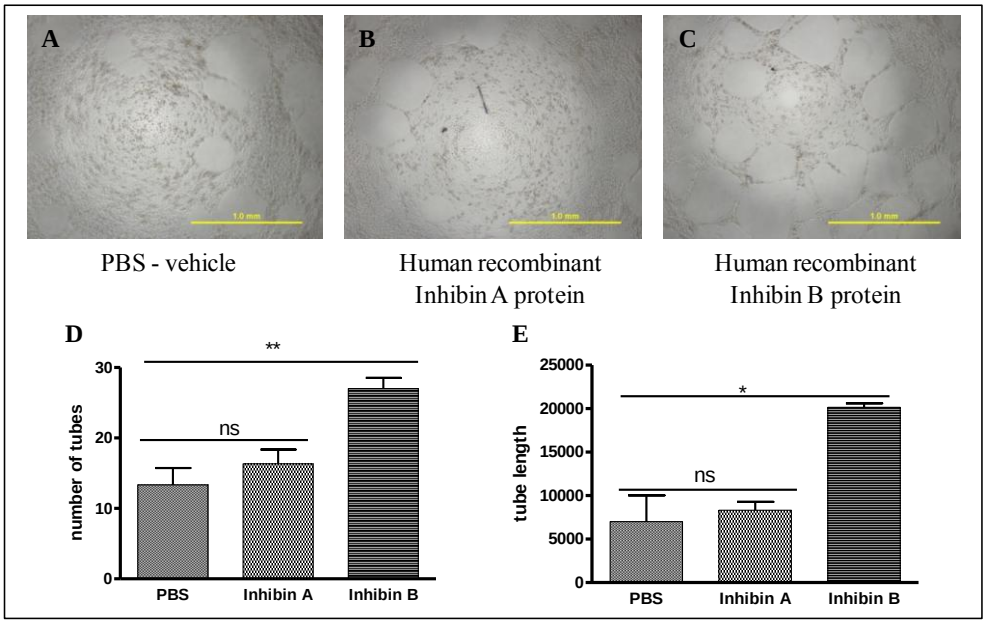

Fig 3 Human recombinant Inhibin B increases LEC tubes formation. Lymphatic endothelial cells (LEC) were seeded in matrigel coated 96 well plates in the presence of PBS vehicle control , 20µg/ml Inhibin A and Inhibin B. (A-C) show LEC tube formation under the different treatments. (D) Difference in tube formation. (E) Difference in length of the tubes. * p 0.01 – 0.05, ** p 0.001 – 0.01, and no significant (ns) difference between PBS vehicle control and recombinant inhibin protein. Data shown as mean ± standard error of the mean.

To determine if media from INHA over-expressing PC3 cells (P20) and EV control (P128) had any effect on LEC tube formation and tube length, we seeded LEC in either normal media or conditioned media obtained from P20 or P128. As shown in Fig 4A-C, LEC in the presence of conditioned media from P20 cells formed more tubes compared to the P128 or normal media. Quantitative analysis of the tubes showed a significant increase in tube formation ($p=0.0001$) and length ($p=0.0002$) in LEC with media from P20 compared to LEC with normal media.

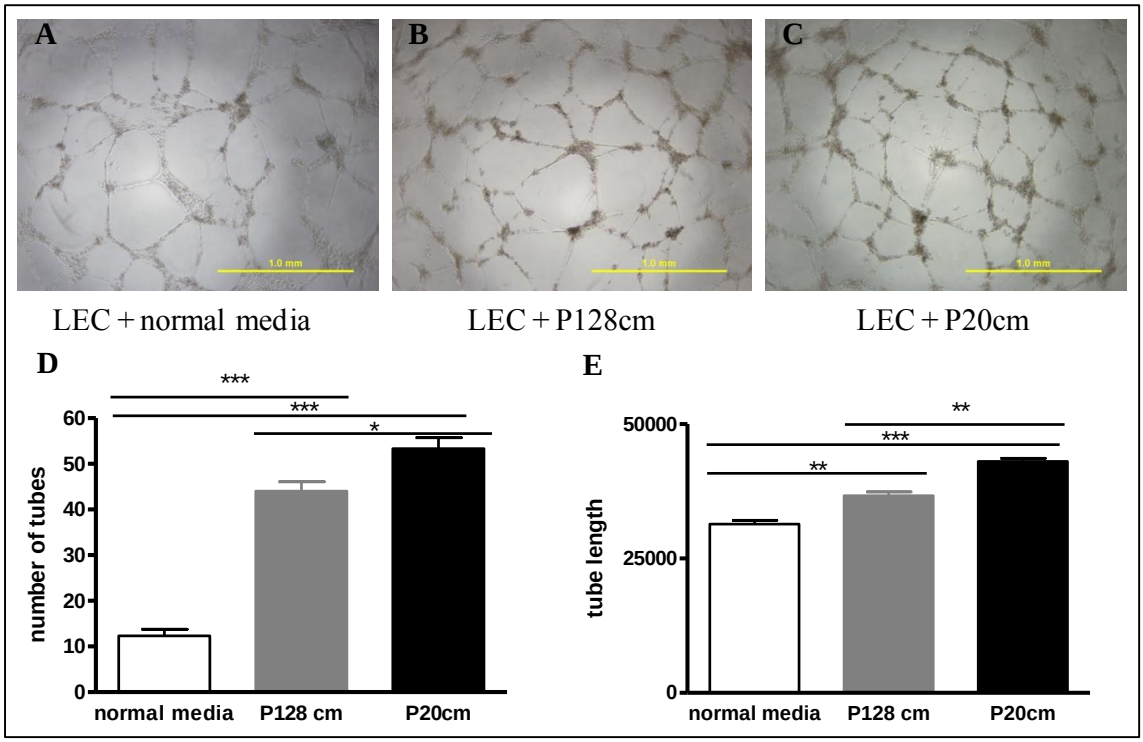

Fig 4 Conditioned media from INHA over-expressing (P20) cell line increases LEC tubes formation. Lymphatic endothelial cells (LEC) were seeded in matrigel coated 96 well plates in the presence of (A) normal media, (B) conditioned media from P128 clone (C) conditioned media from P20 clone. (D) Difference in tube formation. (E) Difference in length of the tubes. * p 0.01 – 0.05, ** p 0.001 – 0.01, *** $p < 0.001$ and no significant (ns) difference between LEC in the presence of normal media, P128cm and P20cm. Data shown as mean ± standard error of the mean.

Furthermore, to determine if in the presence of INHA over-expressing PC3 cells (P20) and EV control (P128), LECs formed tube like structures we co-cultured the two cells types. As shown in Fig 5, LEC were able to form tubes in the presence of P20 cells but not P128 cells.

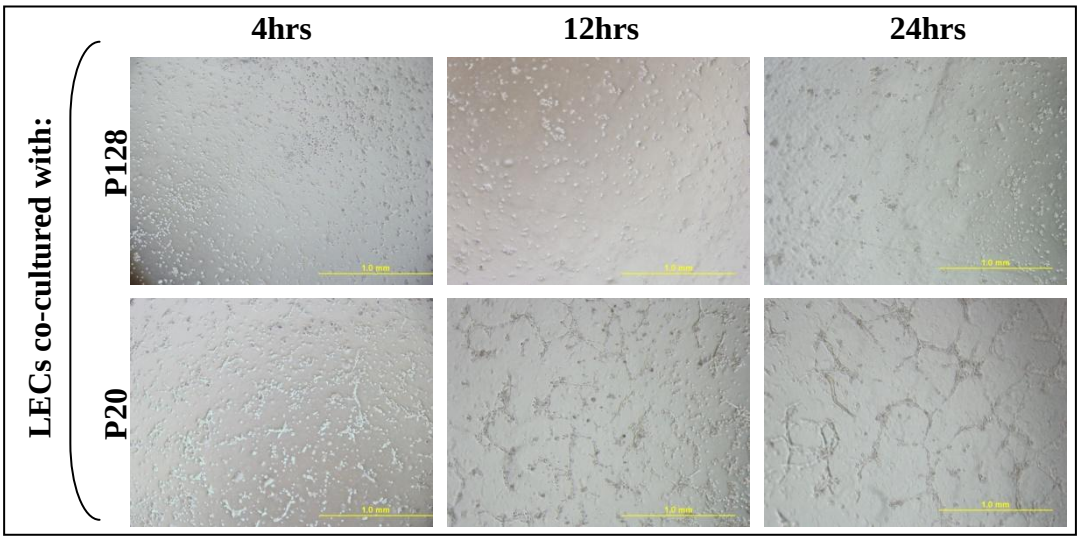

Fig 5 INHA over-expressing PC3 cells co-cultured with LEC form tubes. PC3 with/without INHA expression were co-cultured with LEC in matrigel coated 96 well plates. In the presence of LECs no tube formation was evident in wells with P128 clone but tubes were present in the wells with P20 clone.

Task 2: To determine the mechanism through which INHA may promote tumor growth and metastasis (Months 3 – 24).

a. ELISAs for VEGF-C and VEGF-D expression at the protein level will confirm changes in INHA over-expressing PC3 cells and empty vector (EV) transfected PC3 cells.

We have completed the aims of Task 2a during the first six months of the project. The observed increase in LVD in INHA-positive PC3 tumors suggested that the metastatic spread of the cancer cells from the primary tumor site to the LNs occurs through the process of lymphangiogenesis. Members of the vascular endothelial growth factor (VEGF) family, VEGF-C, VEGF-D and more recently VEGF-A, have been associated with lymphangiogenesis, mediating their effects through vascular endothelial growth factor receptors 2 and 3 (VEGF R2 and VEGF R3) (3-5). Our preliminary data showed that there was no change in VEGF-D mRNA levels in INHA over-expressing PC3 cells compared their EV controls, therefore it was decided not to analyze VEGF-D expression any further. However, we went on to determine the expression of VEGF-A and VEGF-C protein by ELISA, in INHA- and EV-transfected clones *in vitro*. VEGF-A mRNA levels were also determined. For detailed description of the methodology see Appendix 1.

VEGF-A ($p = 0.0002$) and VEGF-C ($p = < 0.0001$) mRNA levels were significantly increased in INHA over-expressing PC3 cells (Table 1). Secreted VEGF-C protein levels were significantly increased ($p = 0.0011$) in the INHA over-expressing PC3 clones compared to their EV clones, however there was no significant change in secreted VEGF-A levels (Table 1).

Table 1 Effect of over-expressing INHA on VEGF-A and VEGF-C

	PC3	
	EV clones	**INHA clones**
VEGF-A		
normalised mRNA	0.016 ± 0.002	0.0423 ± 0.006***
protein (µg/µl)		
cell lysate	626.8 ± 25.04	500.9 ± 24.51*
conditioned media	1794 ± 40.31	1712 ± 34.64
VEGF-C		
normalised mRNA	12.76 ± 1.59	34.96 ± 2.76***
protein (µg/µl)		
cell lysate	319.6 ± 49.21	656.7 ± 46.21**
conditioned media	3377 ± 566.0	6892 ± 531.6**

* p = 0.01 - 0.05
** p = 0.01 - 0.001
*** p = < 0.001

b. Microarray technology, siRNA and small inhibiting molecules will comprehensively analyze and identify the pathway(s) inhibin affects in regulating its tumor promoting and pro-metastatic role.

We have completed the overall aim of Task 2b i.e. we have used microarray technology only to identify the pathway(s) inhibin affects in regulating its tumor promoting and pro-metastatic role. This was the first modification to the initial proposal. At the time of the experiment, we decided to use Affymetrix GeneChip Human GENE 1.0ST array (Affymetrix) and TGFß BMP Signaling Pathway Oligo GEArray (Superarray) to get more cost effective, efficient and robust results instead of using several different signal pathway arrays.

Briefly, INHA and EV transfected PC3 cells were cultured using standard techniques. These cells were then prepared for analysis on the gene arrays according to the manufacturer's instructions. The TGFß BMP Signaling Pathway array experiments were performed at Monash. The Affymetrix GeneChip Human GENE 1.0ST array experiments were performed and analyzed in collaboration with Dr Robin Anderson and Dr Bedrich Eckhardt at the Cancer Biology Group, Peter MacCallum Cacner Centre, Melbourne, Australia. Table 2 shows only the most significant gene changes.

To specifically identify changes in the TGFβ pathway, the Affymetrix data was further analyzed using Ingenuity System. This identified ERK/MAPK pathway to be altered in our INHA-over-expressing PC3 cells compared to the EV cells. Analysis of the TGFβ BMP array also revealed changes in MAPK gene expression and few other genes that may link INHA expression to its tumor promoting role.

In consultation with our associate investigators and collaborators it was decided to use an alternate but more informative method to confirm the pathway inhibin affects in

regulating its tumor promoting and pro-metastatic role. This was the second modification to the initial proposal. As evident in our publication (Appendix 1) we have also demonstrated a tumor and metastasis suppressive effect of INHA in INHA over-expressing LNCaP PCa cell line. LNCaP cell line is androgen-dependent and less metastatic PCa cell lines. In order to demonstrate that the change in the ERK/MAPK pathway is specific to the role of INHA as a pro-tumorigenic and pro-metastatic factor (in INHA over-expressing PC3 cells), we performed the same gene array experiments and analysis on INHA over-expressing LNCaP cells and its EV controls.

Analysis of the array data using the Ingenuity System showed no change in the ERK/MAPK pathway in INHA over-expressing LNCaP cells (Table 3). This suggests that the ERK/MAPK pathway is specific to the pro-tumorigenic and pro-metastatic role of INHA.

The changes to the initial proposal have been beneficial to the overall goal of the project. The information from the gene arrays has not only successfully addressed the aim of this Task "identify pathway(s) inhibin affects in regulating its tumor and pro-metastatic role" but has provided data that can be mined to do in depth studies into why the role of INHA switches from a tumor suppressor to a pro-tumorigeneic and pro-metastatic factor during PCa progression. Extensive experiments in this area using siRNA, small inhibiting molecules and transgenic animal models may result in identifying key genes that can be targeted to reduce tumor and metastasis in INHA over-expressing PCa cells. The above data and future studies associated with it will form the basis for new future grant applications.

c. RNA interference technology via a viral delivery system will be used to down-regulate INHA and VEGF-C expression in INHA over-expressing cells and orthotopic inoculation of these cells in mice will show and confirm that INHA stimulated VEGF-C is responsible for increased in LVD and increased incidence of LN metastasis. We project to use 100 male SCID mice for this study (10 mice per group).

We have completed Task 2c. Before studies on mice can begin, we were required to obtain Animal Ethics approval from Monash Medical Centre Animal Ethics Committee to use mice for this project. The approval was granted (Application number: MMCA 2006/45) on 20 March 2007 [see appendix 4]. We have successfully established viral expression and delivery system in our lab. However, there was significant delay towards commencing the main experiment. While we were able to produce virus, we had difficulty infecting our PCa cells and therefore creating knock-down clones. After several attempts it was decided to use traditional transfection protocols to produce the knock-down clones.

The shRNA plasmids for INHA were purchased from Sigma-Aldrich while the shRNA for VEGF-C was provided by Richard Hynes, Cambridge (6). To date we have successfully created INHA knock-down clones and INHA expression levels were

Table 2 Difference in gene expression in INHA over-expressing PC3 cells compared to EV cells.

Changes in gene expression using TGF BMP signaling pathway array

Gene symbol	Description	fold difference	p value
Genes over-expressed			
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	2.48	0.028
TGIF1	TGFB-induced factor homeobox 1	2.07	0.005
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	2.05	0.064
TGFB2	Transforming growth factor, beta 2	2.00	0.043
MAP3K7IP1	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	1.78	0.003
INHBA	Inhibin, beta A (activin A, activin AB alpha polypeptide)	1.58	0.419
SMURF1	SMAD specific E3 ubiquitin protein ligase 1	1.57	0.036
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	1.54	0.106
Genes under-expressed			
RUNX2	Runt-related transcription factor 2	0.64	0.202
LASS1	LAG1 homolog, ceramide synthase 1 (S. cerevisiae)	0.42	0.186
GDF3	Growth differentiation factor 3	0.40	0.158

Changes in gene expression using Affymetrix array

Gene symbol	Description	fold difference	p value
BEX1 (includes EG:55859)	brain expressed, X-linked 1	9.35	32.78
INHA	inhibin, alpha	4.10	3.55
DPP4	dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	4.90	2.48
TSPAN12	tetraspanin 12	1.53	2.19
SNX16	sorting nexin 16	2.19	2.03
ASRGL1	asparaginase like 1	2.04	2.02
PLP2	proteolipid protein 2 (colonic epithelium-enriched)	1.56	1.89
TRPS1	trichorhinophalangeal syndrome I	1.55	1.87
PAPSS1	3'-phosphoadenosine 5'-phosphosulfate synthase 1	1.53	1.83
PLCB4	phospholipase C, beta 4	2.83	1.79
SPATA6	spermatogenesis associated 6	1.52	1.77
TUBA1A	tubulin, alpha 1a	3.99	1.75
ERV3 (includes EG:2086)	endogenous retroviral sequence 3 (includes zinc finger protein H-plk/HPF9)	1.67	1.74
LOC100133941	CD24 molecule	6.66	1.70
LOC158160	hydroxysteroid (17-beta) dehydrogenase 7 pseudogene 2	1.59	1.67
NRIP1	nuclear receptor interacting protein 1	1.54	1.63
MYEF2	myelin expression factor 2	3.82	1.62
CACNB3	calcium channel, voltage-dependent, beta 3 subunit	2.00	1.60
ZNF816A	zinc finger protein 816A	1.77	1.59
SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	1.63	1.54
GRAMD3	GRAM domain containing 3	1.61	1.52
SPECC1	sperm antigen with calponin homology and coiled-coil domains 1	1.60	1.51
STARD4	StAR-related lipid transfer (START) domain containing 4	2.29	1.51
LOC646853	hypothetical LOC646853	1.72	1.50
PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium-dependent)	-3.45	-1.54
HOXB9	homeobox B9	-1.62	-1.55
LCN2	lipocalin 2	-2.19	-1.57
ADCY1	adenylate cyclase 1 (brain)	-1.52	-1.61
WSB1	WD repeat and SOCS box-containing 1	-1.58	-1.62
ERO1L	ERO1-like (S. cerevisiae)	-2.33	-1.63
NMD3	NMD3 homolog (S. cerevisiae)	-1.62	-1.68
ADM	adrenomedullin	-1.53	-1.71
HK2	hexokinase 2	-1.84	-1.87
RAB31	RAB31, member RAS oncogene family	-2.19	-2.07
PXDN	peroxidasin homolog (Drosophila)	-1.78	-2.26
TFPI	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	-1.55	-2.31

confirmed by radio-immunoassay Table 4. However, even after several attempts we were not able to create VEGF-C knock-down clones. Since role of VEGF-C in promoting growth of lymphatic vessels into and around tumors in animal models its association with metastatic spread to the lymph nodes and sometimes to distant organs has been well documented (2, 7, 8) and the technical difficulties we were facing, it was decided not to continue with attempting to knockdown VEGF-C in INHA over-expressing PC3 cells.

As shown in Table 4 our INHA knock-down (KD) experiments resulted in n=4 INHA KD clones, n=1 non-target (NT) control and n=2 EV (0.1) controls. Unfortunately, n=2 INHA KD clones (P20/904/1 and P20/904/2) failed to grow in culture. This left us with

Table 3 Difference in gene expression in INHA over-expressing LNCaP cells compared to EV cells.

Changes in gene expression using TGF BMP signaling pathway array			
Gene symbol	**Description**	**fold difference**	**p value**
Genes over-expressed			
RUNX2	Runt-related transcription factor 2	2.35	0.18
LASS1	LAG1 homolog, ceramide synthase 1 (S. cerevisiae)	2.16	0.24
PDGFB	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	1.97	0.13
GDF3	Growth differentiation factor 3	1.95	0.21
SOX4	SRY (sex determining region Y)-box 4	1.72	0.05
JUNB	Jun B proto-oncogene	1.65	0.01
Changes in gene expression using Affymetrix array			
Gene symbol	**Description**	**fold difference**	**p value**
BEX1 (includes EG:55859)	brain expressed, X-linked 1	32.78	0
INHA	inhibin, alpha	3.55	0
DPP4	dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	2.48	0
SLC2A12	solute carrier family 2 (facilitated glucose transporter), member 12	2.27	0.005
TSPAN12	tetraspanin 12	2.19	0
ZNF323	zinc finger protein 323	2.16	0.029
MAST4	microtubule associated serine/threonine kinase family member 4	2.07	0.03
SNX16	sorting nexin 16	2.03	0.001
ASRGL1	asparaginase like 1	2.02	0.002
DGKH	diacylglycerol kinase, eta	-2.01	0.01
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	-2.01	0
RAB31	RAB31, member RAS oncogene family	-2.07	0
LCP1	lymphocyte cytosolic protein 1 (L-plastin)	-2.10	0.014
PXDN	peroxidasin homolog (Drosophila)	-2.26	0.001
TFPI	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	-2.31	0.001
P2RY5	purinergic receptor P2Y, G-protein coupled, 5	-2.46	0.025
RELN	reelin	-3.44	0.001

INHA KD clones generated from different INHA gene target sites (904 and 905). Before proceeding to costly and extensive animal studies, we conducted preliminary *in vitro* cell viability/proliferation assay using the Cell Titer 96®AQueousOne solution cell proliferation assay (Promega, Hawthorn, VIC, Australia) to determine the functional consequences of knocking-down INHA in INHA over-expressing PC3 cells. We have previously demonstrated that over-expressing INHA in PC3 cell line significant increased its proliferative capability (Appendix 1). As shown in Figure 6 only INHA KD clone P20/905/1 demonstrated a significant reduction (p=0.0004) in cell proliferation while the NT and EV controls were similar to their parental P20 (INHA over-expressing PC3). Due to the failure in generating at least n=2/3 INHA KD clones (for statistical significance) that demonstrated similar growth characteristic as the parent PC3 cells, it was deemed unethical to proceed with the *in vivo* animal studies that would results in non-publishable and statistically non-significant data.

Table 4 INHA knock-down clones selected for further analysis

PC3 cells	Inhibin (ng/ml)
INHA knock-down clones	
P20/904/1	<0.12
P20/904/2	<0.12
P20/904/3	<0.12
P20/905/1	<0.12
control clones	
P20/NT/5	0.63
P20/0.1/5	0.31
P20/0.1/6	0.34
parental INHA-over-expressing cells	
P20	0.30

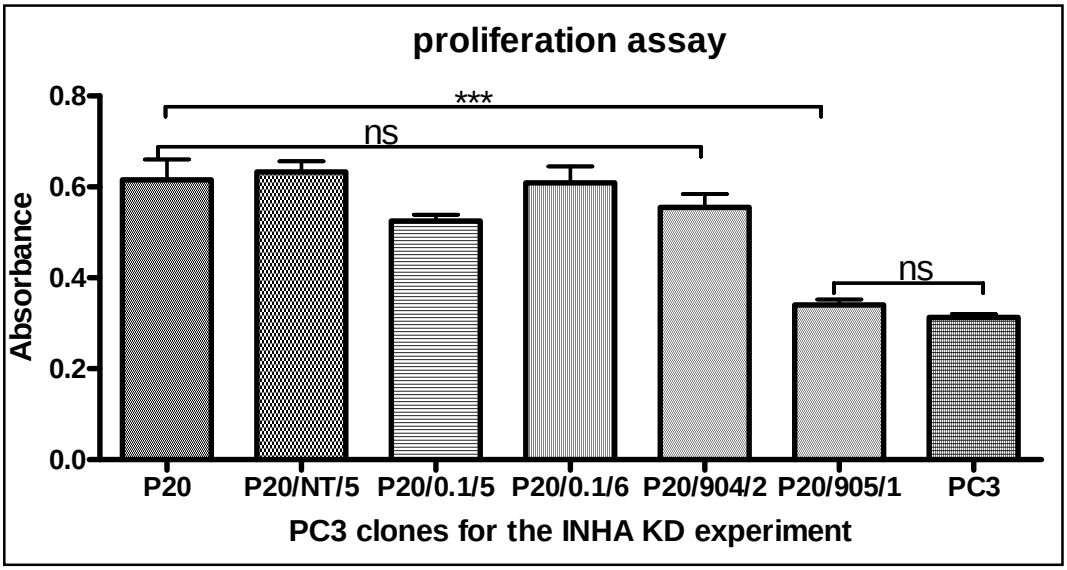

Fig 6 Proliferation assay of INHA knockdown clones in INHA over-expressing cells. Cells were seeded overnight into 96-well plates and cell proliferation assay conducted. INHA over-expressing PC3 clone (P20), non-target control (NT/5), EV controls (0.1/5, 0.1/6), INHA KD clone (904/2, 905/1) and parental PC3 cells. NT and EV controls have similar proliferative capability as their P20 parent line. Only INHA KD clone 905/1 showed significant reduction in proliferation which was similar to the parental PC3 cell lines. Data shown as mean ± standard error of the mean.

d. Orthotopic inoculation of INHA and empty vector (EV) transfected PC3 cells followed regular injections of neutralizing antibodies will be used to block VEGF receptor signaling in the cells. This will show that by blocking VEGF receptor signaling we can reduce LVD and the incidence of LN metastasis. We project to use 60 male SCID mice for this study (10 mice per group).

We have completed the aims of Task 2d. There had been a delay towards this aim since we were unable to obtain the neutralizing antibodies (VEGF R2 and VEGF R3) to block VEGF receptor signaling in the cells as originally planned. To minimize this having an

effect on this project and delaying the experiments further we formed a collaborative research with Schering AG, Corporate Research Oncology who has sent us PTK/ZK, a compound known to specifically block VEGF receptor signaling (9). PTK/ZK has successfully been used in both animal experiments (9) and human clinical trial for advanced colorectal cancer, acute myeloid leukemia and liver metastases (10, 11).

Before this work begins, we were required to obtain Animal Ethics approval from Monash Medical Centre Animal Ethics Committee to use mice for this project. The approval was grants (Application number: MMCA 2006/45) on 20 March 2007 [see appendix 4]. The use of this compound instead of the neutralizing antibodies also reduced the number of mice to be used for this aim. We used 40 male SCID mice for this study (10 mice per group).

Method: INHA- and EV-transfected cells were cultured using standard tissue culture techniques. These cells were injected into the prostate of 6 – 8 week old male SCID mice (10^5 cells/ injection) with the aim of forming tumors. Upon establishment of tumors, PTK/ZK was administrated orally per day for a period of 30-35 days. Each of 4 groups included 10 mice (Table 5).

Table 5 Animal groups for AIM 2d
EV transfected PC3 cell line (P128)
INHA transfected PC3 cell line (P20)
INHA transfected PC3 cell line (P20) under PTK/ZK treatment
EV transfected PC3 cell line (P128) under PTK/ZK treatment

The mice were monitored over a period of 8 weeks after which the primary tumors and regional LNs were collected. Using standard histopathological techniques collected tissues were sectioned and subjected to immunohistochemical (IHC) and stereological analysis and the incidence of LN metastasis were determined.

Expected results:
PC3 cell line produce VEGF family members, therefore blocking VEGF-R using PTK/ZK treatment should reduce tumor growth and LN metastasis. Our P128 model is an EV clone, therefore a representative of PC3 parental line. We expect mice with P128 tumors and treated with PTK/ZK to have small tumors and low incidence of LN metastasis compared to those treated with vehicle. As for P20, we expect similar outcome which may or may not be significant due to significant increase in VEGF-A and VEGF-C post INHA-transfection (Table 1). Furthermore, vehicle treatment P128 and P20 tumors should have similar results as show in Fig 1.

Experimental results:
There are several inconsistencies in the results from the above experiment. The tumor take, tumor weight and incidence of LN metastasis of P128 and P20 tumors (Fig 6) are not consistent to previous observed results (Fig 1).

Tumor take: We have previously shown that tumor take was 100% in both mice injected with P20 (INHA++) and those injected with P128 (INHA-- clone) (ref to Fig 1). The current results from this aim show that the tumor take is low in mice injected with P20 compared to P128 (Fig 6A).

Tumor weight: Furthermore, we have previously shown that P20 (INHA++) had significantly larger tumors than P128 (INHA-- clone) (ref to Fig 1), hence INHA over-expression in PC3 cells was tumor promoting. In the current experiment we have observed smaller tumors in mice injected with P20 compared to those injected with P128 (Fig 6B).

LN metastasis: Similarly we have previously shown that the incidence of LN metastasis to be higher in mice injected with P20 (INHA++) compared to those injected with P128 (INHA-- clone) (ref to Fig 1). The current results show that the incidence of LN metastasis is low in mice injected with P20 compared to P128 (Fig 6C).

Despite the inconsistencies, PTK/ZK treatment of mice with P128 tumors was successful. These mice showed reduction in tumor take, the tumors were smaller in size and there was also a reduction in the incidence of LN metastasis compared vehicle treated mice. From this part of the study we can conclude that PTK/ZK has the potential to be used in the treatment of PCa patients to reduce the spread of cancer cells from the primary prostate tumor site to the LNs.

As mentioned above P20 tumors did not respond as expected. There may be two possible explanations for this:

1. changes to the tumorigenic properties of P20 while in culture prevented it from being more aggressive than P128
2. PTK/ZK treatment failed to block tumor growth and metastasis of P20 tumors because the previously observed outcomes (not reproducible here) are independent of VEGF family driven metastasis.

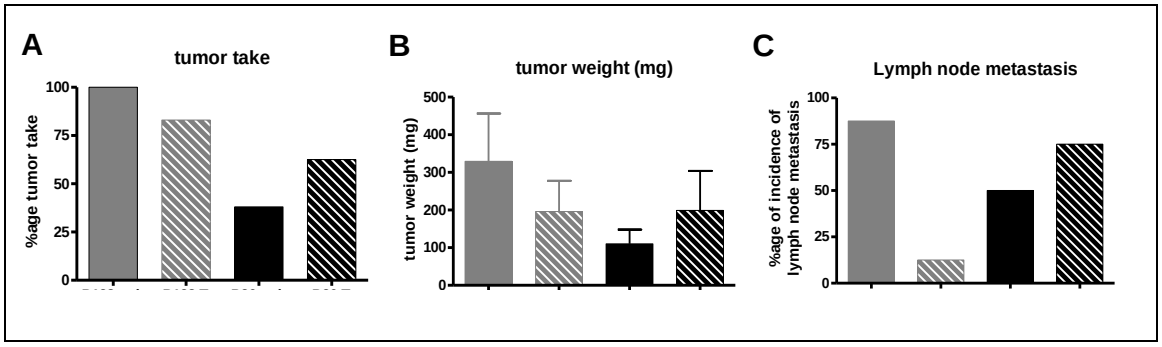

Fig 6 Effect of blocking VEGF receptor signaling in PC3 cell lines. The bars represent: EV-transfected PC3 clones in grey, INHA-transfected clones in black. Data shown as mean ± standard error of the mean.

Task 3: To determine the utility of INHA for the diagnosis of patients with highly aggressive and/ or metastatic PCa (Months 3-8)

a. Collection of archival human prostate tissue of at least 50 men who under radical prostatectomy. Tissues will be collected from patient who had organ-confined disease and those with lymph node metastasis.

We have completed the aims of Task 3a during the first six months of the project. Before this work begins, we were required to obtain Human Ethics approval to access archival human prostate tissues from patients who under went radical prostatectomy surgery. The approval was granted from Monash University Standing Committee on Ethics in Research Involving Humans (Approval Number: CF07/0854 – 2007/0223HT) on 16 May 2007 [see appendix 5]. We collected archival tissues from patients who had organ-confined disease and those with lymph node metastasis.

b. Immunohistochemistry for INHA, VEGF-C and D2-40 will show the utility of INHA as a diagnostic or prognostic marker for PCa patients.

We have completed the aims of Task 3b during the first six months of the project. However, in the last 2-3 months we have re-evaluated the data for further analysis (see below). To reduce wastage of precious human prostate tissues it was decided to use a cohort of patient tissues that have already being evaluated for clinicopathological characteristics, VEGF-C expression and LVD (using D2-40) (1, 12) by our collaborating investigator; Elizabeth Williams. A number of independent studies have shown increases in INHA expression to be associated with PCa progression (13, 14). To determine if INHA expression can be correlated to lymph node status we used tissues from a cohort of PCa patients who had organ-confined disease and those who had lymph node metastasis. We obtained 20 radical prostatectomy specimens were from patients with organ-confined disease, while the remaining 16 specimens were from patients with LN metastases. The PO#12 antibody was used to determine the expression pattern of INHA in the prostate tissues. For detailed description of methodology see Appendix 1. An example of the INHA staining and scoring is provided in Fig 7.

Initial analysis of the immunostaining revealed significant increase in INHA staining in normal epithelial and intraepithelial neoplasia (PIN) regions with no change in the staining in the cancer regions of the prostate tumor tissues in patients with LN metastasis compared to patients with organ-confined disease (Fig 8A). However, there was no significant change in overall INHA expression in patients with LN metastasis compared to patients with organ-confined disease (Fig 8B).

We have recently re-evaluated the immunostaining and conducted a cross-sectional study to determine a link between INHA expression and a number of clinicopathological parameters including Gleason score, surgical margin, extracapsular spread, lymph node status and VEGF-R3 expression which are well established prognostic factors of PCa (15-19). For detailed description of methodology and statistical analysis see Appendix 1.

The immunostaining revealed differential expression of INHA in benign epithelial, G3/G4 cancer regions as well as in the stroma of primary PCa tissues from patients with organ-confined disease and those with metastasis to the lymph nodes (Fig 9). Association between clinicopathological prognostic factors and INHA expression are shown in Table 6. Elevated expression of INHA in the benign regions of the primary PCa tissues showed a higher relative risk in PCa patients been positive for extracapsular spread ($p = 0.01$). Similarly, elevated expression of INHA in the stroma of the primary PCa tissues showed a higher risk of PCa patients been positive for extracapsular spread ($p = 0.0011$), positive for surgical margins ($p = 0.0006$), positive for VEGF-R3 expression ($p = 0.00067$) and positive for lymph node metastasis ($p < 0.0001$). Further analysis showed that there was a significant increase in INHA staining in benign ($p = 0.018$) and stromal ($p < 0.0001$) regions but not in G3/G4 cancer regions in tissues from patients with lymph node metastasis compared to patients with organ-confined disease (data not shown).

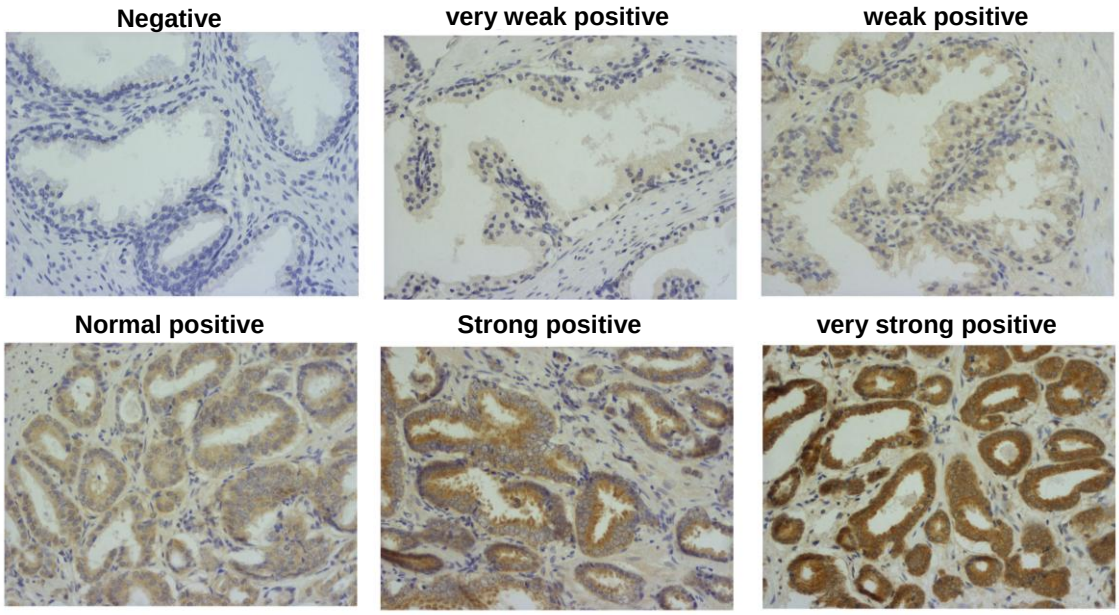

Fig 7 Example of immunohistochemistry staining intensity used to evaluate the intensity of INHA staining in the prostate tissues. Each immunostained tissue section was assessed and staining intensity in the different regions and grades of tumor was scored as following: -: negative (0); +/-: very weak positive staining (0.5); +: weak positive staining (1); ++: normal positive staining (2); +++: strong positive staining (3); ++++: very strong positive staining (4). Intensity of INHA staining in normal epithelial and intraepithelial neoplasia (PIN) and cancer regions (Gleason grade G1-G5) were analyzed to determine the pattern of INHA expression in the prostate tissues from patients with organ-confined disease and those with LN metastasis.

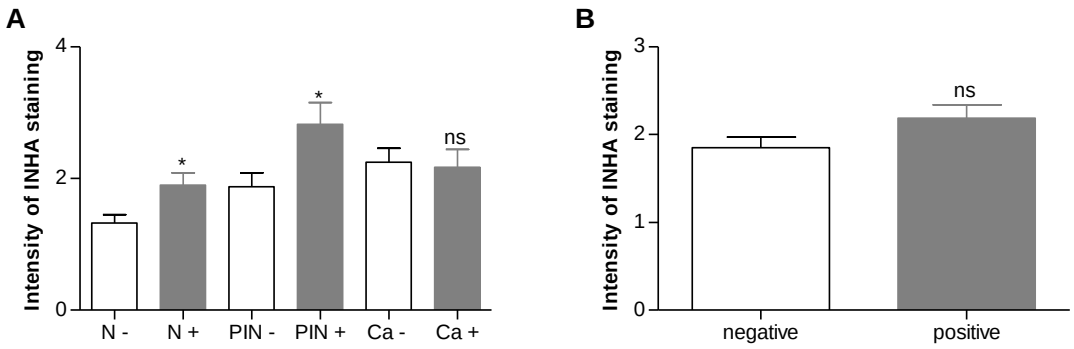

Fig 8 INHA staining in PCa patients with organ confined and metastatic disease. *A*, INHA immunostaining intensity in normal epithelial (N), PIN and cancer regions (Ca) was compared in patients with organ-confined prostate cancer (-) and those with metastasis to the lymph nodes (+). * p 0.01 – 0.05 and no significant (ns) difference between the respective regions in organ confined and metastatic disease. *B.* Overall, there was no significant difference in INHA intensity in tissues from patients with organ-confined prostate cancer (negative) and those with metastasis to the LN (positive).

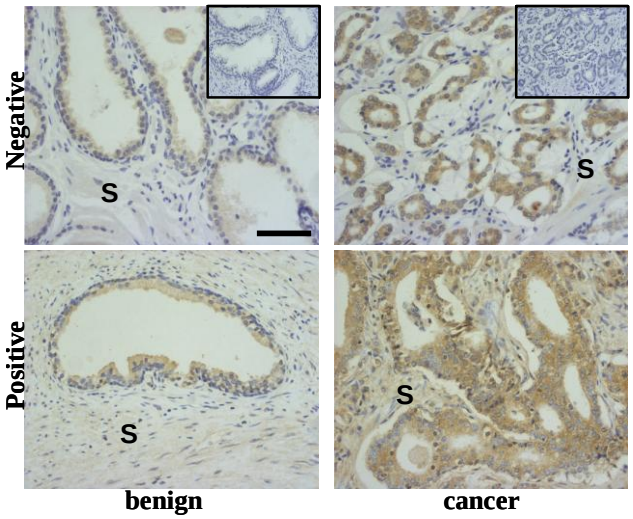

Fig 9 INHA expression in clinical specimens and its association to prostate disease. Immunohistochemical staining of INHA in primary prostate tumors from PCa patients with organ confined (negative) and metastatic disease (positive). INHA immunostaining intensity in benign epithelial, cancer region (G3/G4) and stromal regions (S) are shown. Insert shows IgG control. Bar 200μM

Table 6 Relationships between the expression of INHA and clinicopathological parameters in prostate adenocarcinoma (n = 37)

Parameters	No. of specimens	benign regions			Cancer regions (G3/G4)			stromal regions		
		mean intensity	Relative risk (95% CI)	p value	mean intensity	Relative risk (95% CI)	p value	mean intensity	Relative risk (95% CI)	p value
combined Gleason grade										
6	16	0.7	n/a		1.88	n/a		0.30	n/a	
7	14	1.51	n/a		2.21	n/a		1.21	n/a	
≥8	7	1.29	n/a		1.99	n/a		2.00	n/a	
extracapsular spread										
Positive	24	1.43			2.13			1.41		
Negative	13	0.65 (reference)	2.07 (1.04 - 4.13)	0.01^	1.87 (reference)	1.27 (0.76 - 2.11)	ns^	0.08 (reference)	2.55 (1.39 - 4.65)	0.0011^
surgical margins										
Positive	16	1.15			1.84			1.47		
Negative	21	1.12 (reference)	1.15 (0.68 - 3.08)	ns^	2.18 (reference)	0.71 (0.32 - 1.53)	ns^	0.50 (reference)	4.75 (1.62 - 13.93)	0.0006^
VEGF-R3+ vessels										
Positive	18	1.29			2.07			1.53		
Negative	19	0.97 (reference)	1.27 (0.62 - 2.60)	ns^	2.00 (reference)	1.20 (0.0 - 2.39)	ns^	0.33 (reference)	2.85 (1.28 - 6.37)	0.0067^
Lymph node metastasis										
Positive	16	1.41			2.06			1.93		
Negative	21	1.01 (reference)	1.62 (0.67 - 3.97)	ns^	2.00 (reference)	0.84 (0.48 - 1.46)	ns^	0.15 (reference)	13.22 (1.94 - 90.00)	p < 0.0001^

Abbreviations: VEGFR, vascular endothelial growth factor receptor; n/a, not applicable; ns, not significant; CI, confidence interval

^ Fisher's exact test

RSEARCH ACCOMPLISHMENTS

- Demonstrated that over-expression of INHA enhanced metastatic ability of tumor cells *in vivo*.
- Gained Human Ethics approval for collection of fresh and archival human prostate tissues.
- Demonstrated that recombinant Inhibin B protein and not Inhibin A promotes lymphatic endothelial cell (LEC) tube formation.
- Demonstrated the LECs cultured with either conditioned media from INHA over-expressing PC3 cells or with INHA over-expressing PC3 cells induced significant increase in tube formation compared to the controls.
- Determined VEGF-A and VEGF-C expression at the protein level in INHA over-expression PC3 cells and the controls.
- Identified a possible signaling pathway inhibin affects in regulating tumor growth and metastasis.
- Created INHA knock-down clones.
- Obtained PTK/ZK, a compound which blocks VEGF family member signaling by blocking their receptor activity.
- Demonstrated the PTK/ZK treatment of mice with tumors can reduce tumor take, tumor size and incidence of metastasis to the LNs.
- Determined INHA expression pattern in PCa patients with organ-confined and metastatic disease.
- Demonstrated that increase in INHA staining in tissues from PCa patients is associated with a higher risk of surgical margin, extracapsular spread, lymph node status and VEGF-R3 expression which are well established prognostic factors of PCa.

REPORTABLE OUTCOMES

	Reportable outcomes that have resulted from this research:
Manuscripts	**Balanathan P, Williams ED, Wang H, Pedersen JS, Horvath LG, Achen MG, Stacker SA, Risbridger GP** 2009 Elevated level of inhibin-alpha subunit is pro-tumourigenic and pro-metastatic and associated with extracapsular spread in advanced prostate cancer. Br J Cancer 100:1784-1793 (Appendix 1)
Abstracts and presentations	Preetika Balanathan, Elizabeth D Williams, Hong Wang, Marc G Achen, Steven A Stacker, Gail Risbridger (2008) **Shift in the tumor suppressive activity of inhibin-α subunit during the transition from androgen-dependent to androgen-independent prostate cancer** TGFβ family in Homeostasis and Disease – Keystone Symposia, Santa Fe New Mexico, USA (poster presentation). [see appendix 6]

	Preetika Balanathan, Elizabeth D Williams, Hong Wang, Marc G Achen, Steven A Stacker, Gail Risbridger (2008) **Tumor suppressive activity of inhibin-α subunit is altered during the transition from androgen-dependent to androgen-independent prostate cancer** Lorne Cancer, Victoria, Australia (poster presentation). [see appendix 7] Preetika Balanathan (2008) **New action of inhibin-α in advanced prostate cancer** GlaxoSmithKline Post Graduate Support Grant award presentation day, Melbourne, Victoria. Preetika Balanathan, Elizabeth D Williams, Hong Wang, Eckhardt Bedrich, John S Pedersen, Lisa G Horvath, Marc G Achen, Steven A Stacker, Robin Anderson, Gail P Risbridger (2009) **Elevated level of inhibin-α subunit is pro-tumourigenic and pro-metastatic and associated with extracapsular spread in advanced prostate cancer** Endocrine Society of Australia Annual Meeting, Adelaide, South Australia, Australia [see appendix 11]
Patents and licenses applied for and/or issued	Nil
Degrees obtained that are supported by this award	Nil
Development of cell lines, tissues or serum repositories	Nil
Informatics such as databases and animal models, etc	Nil
Funding applied for based on work supported by this award	GlaxoSmithKline (GSK) Post Graduate Support Grant for 2008 [see appendix 8] ANZ Philanthropy Trust Fund Grant 2008 [see appendix 9] Keystone Symposia – Travel scholarship [see appendix 10]
Employment or research opportunities applied for and/or received based on experience/training supported by this award	Nil

CONCLUSIONS

In summary, we have made significant progress towards understanding the role of INHA in advanced PCa. We have demonstrated increased tumor size and increased metastasis to the LNs by INHA over-expressing PC3 cells compared to the controls. The increase in metastasis was further evident by increase in total LVD and lymphatic invasion which was accompanied by increase in VEGF-C expression. *In vitro* tube formation assays have implicated Inhibin B as the form of inhibin responsible for the increase in tube formation in the experiments.

Using clinical specimens we have been able to determine that there is evidence of increased INHA expression in benign epithelial and stromal regions in tissues from PCa patients. This increase in INHA expression was significantly associated with higher risk of surgical margin, extracapsular spread, lymph node status and VEGF-R3 expression which are well established prognostic factors of PCa. Our work on understanding the mechanism suggests a role of ERK/MAPK signaling pathway through which INHA promotes tumor growth and metastasis. This study is the first to demonstrate a pro-tumorigenic and pro-metastatic function for INHA associated with androgen-independent stage of metastatic prostate disease. Our results also suggest that INHA expression in the primary prostate tumor can be used as a predictive factor for prognosis of PCa. The outcomes of these experiments have contributed significantly to our understanding of the role of INHA in the process of prostate carcinogenesis.

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APPENDIX 1: MANUSCRIPT

British Journal of Cancer (2009), 1 – 10

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Full Paper

Elevated level of inhibin-α subunit is pro-tumourigenic and pro-metastatic and associated with extracapsular spread in advanced prostate cancer

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The biological function of inhibin-α subunit (INHα) in prostate cancer (PCa) is currently unclear. A recent study associated elevated levels of INHα in PCa patients with a higher risk of recurrence. This prompted us to use clinical specimens and functional studies to investigate the pro-tumourigenic and pro-metastatic function of INHα. We conducted a cross-sectional study to determine a link between INHα expression and a number of clinicopathological parameters including Gleason score, surgical margin, extracapsular spread, lymph node status and vascular endothelial growth factor receptor-3 expression, which are well-established prognostic factors of PCa. In addition, using two human PCa cell lines (LNCaP and PC3) representing androgen-dependent and -independent PCa respectively, we investigated the biological function of elevated levels of INHα in advanced cancer. Elevated expression of INHα in primary PCa tissues showed a higher risk of PCa patients being positive for clinicopathological parameters outlined above. Over-expressing INHα in LNCaP and PC3 cells demonstrated two different and cell-type-specific responses. INHα-positive LNCaP demonstrated reduced tumour growth whereas INHα-positive PC3 cells demonstrated increased tumour growth and metastasis through the process of lymphangiogenesis. This study is the first to demonstrate a pro-tumourigenic and pro-metastatic function for INHα associated with androgen-independent stage of metastatic prostate disease. Our results also suggest that INHα expression in the primary prostate tumour can be used as a predictive factor for prognosis of PCa.
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Keywords: inhibin-α subunit; prostate cancer; metastasis; androgen independent

Inhibins A and B are members of the transforming growth factor-β (TGFβ) superfamily. Inhibins are heterodimers of an 18 kDa α-subunit disulphide linked to one of two 13 kDa β-subunits (βA and βB) resulting in inhibin A (αβA) and B (αβB) respectively. Primarily of gonadal origin, inhibins regulate pituitary follicle-stimulating hormone secretion by feedback inhibition. In humans, women produce both inhibin A and inhibin B (Welt *et al*, 1999; Welt and Schneyer, 2001), whereas in adult men inhibin B is the primary form (Marchetti *et al*, 2003). Inhibins have also been shown to be expressed in adrenal cortex, pituitary and prostate (reviewed in Risbridger *et al*, 2001). Other endocrine and paracrine functions of inhibins involve regulating members of the TGFβ superfamily, such as TGFβ itself, activins and bone morphogenic protein (BMP), by competing for their receptors (Wiater and Vale, 2003; Farnworth *et al*, 2007). This antagonistic effect of inhibin is amplified in the presence of the inhibin receptor, TGFβ receptor III (TGFβRIII), also known as betaglycan.

The first study that linked inhibin to reproductive cancers showed that serum inhibin increased in women with granulosa cell tumours of the ovary (Lappohn *et al*, 1989) and inhibin currently serves as a robust biomarker for this cancer. A direct biological function for inhibin in carcinogenesis was demonstrated by Matzuk *et al* (1992) when they created the inhibin-α subunit (INHα) knockout mouse. Both sexes developed gonadal sex-cord stromal tumours with high penetrance and developed tumours in their adrenal glands after castration, showing that INHα can act as a tumour suppressor. Subsequent mouse model studies revealed a complex network of interactions involving inhibin, activin and other modifiers in the development and progression of gonadal and adrenal tumours in INHα-deficient mice (reviewed in Risbridger *et al*, 2001). Although it is clear that total inhibin as a serum test has utility in the initial detection and prognosis of certain types of ovarian cancers, the biological function of inhibin in tumourigenesis is far from clear.

We have observed both up- and down-regulation of INHα expression in prostate cancer (PCa) tissues dependent on the stage

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of disease. Studies from our laboratory on metastatic PCa epithelial cell lines demonstrated that high concentrations (400 ng ml^{-1}) of recombinant inhibin A inhibited growth of androgen-responsive LNCaP cells, but not androgen-independent DU145 cells, suggesting that the effect of inhibin may be dependent on cell phenotypes related to specific stages of PCa (McPherson *et al*, 1997). Furthermore, we have shown loss of heterozygosity or epigenetically regulated loss or down-regulation of INHα in PCa patient samples and LNCaP, DU145 and PC3 cell lines (Mellor *et al*, 1998; Schmitt *et al*, 2002; Balanathan *et al*, 2004). Collectively, these data suggest a tumour suppressive function for INHα in the prostate. In contrast, new insights into the role of INHα in the prostate came from a recent study using a large cohort of PCa patient tissues that showed that INHα was frequently over-expressed in high-grade PCa (Risbridger *et al*, 2004a, b). The intensity of INHα immunoreactivity was associated with a higher risk of recurrence of PCa. These variable clinical and experimental observations provide equivocal evidence for a role of INHα in PCa. Thus it was proposed that like TGFβ (Roberts and Wakefield, 2003), INHα has tumour suppressive activity in normal epithelial cells, which changes to tumour promoting in cancer cells (Ball *et al*, 2004; Risbridger *et al*, 2004a).

To date there is no proof that INHα can increase tumourigenesis and metastasis. Thus the primary aim of this study was to examine the pro-tumourigenic and pro-metastatic function of INHα in advanced PCa. Specifically, the expression profile of INHα and clinicopathological parameters were examined in primary PCa tissues including specimens from patients with organ-confined disease and those with metastasis to the lymph nodes. In addition, human metastatic PCa cell lines, LNCaP and PC3, were used in *in vitro* and *in vivo* functional studies to determine the biological function of elevated levels of INHα on migration, invasion, tumour growth and metastasis.

MATERIALS AND METHODS

Analysis of clinical material

Relationship between INHα expression and clinicopathological parameters in primary prostate adenocarcinomas This study was conducted in accordance with Australian National Health and Medical Research Council (NHMRC) guidelines. Archival formalin-fixed paraffin-embedded tissue blocks were retrieved from 37 patients with prostate carcinoma who underwent radical prostatectomy. The clinicopathological characteristics, vascular endothelial growth factor-C (VEGF-C) expression, lymphatic vessel density (LVD) and lymph node status of this cohort have been described previously (Zeng *et al*, 2004, 2005). We conducted a cross-sectional study to determine whether INHα expression was associated with clinicopathological parameters (Gleason score, surgical margins, extracapsular spread, VEGF receptor-3 (VEGFR-3) expression and lymph nodes status) and/or linked to well-established prognostic factors in prostatic adenocarcinoma. The PO 12 antibody (kindly provided by Dr Nigel Groome) was used to determine the expression pattern of INHα in primary prostate tissues as previously described (Risbridger *et al*, 2004b). Each immunostained tissue section was assessed and staining intensity in benign epithelial, cancer (Gleason grade G3/G4) and stromal regions was scored from 0 to 4 with 0 representing negative staining and 4 representing very strong positive staining. The relative risk of PCa patients being positive for the respective parameters was determined.

Stable transfection of LNCaP and PC3 cell lines LNCaP and PC3 were obtained from American Type Culture Collection (Rockville, MD, USA) and routinely cultured as described previously (Balanathan *et al*, 2004). Expression vectors pcDNA3.1 (empty vector, EV) and human *INHα* cDNA subcloned into pcDNA3.1 (pcDNA3.1 (INHα)) were purchased from Invitrogen (Mount Waverley, Victoria, Australia) and prepared for transfection according to the manufacturer's instructions. LNCaP and PC3 cells were transfected using Lipofectamine plus (Invitrogen) and Superfect (Qiagen, Doncaster, Victoria, Australia) respectively according to the manufacturer's instructions. Individual colonies surviving after 2–3 weeks selection were picked and propagated for analysis.

Confirmation of mRNA expression in INHα-transfected LNCaP and PC3 cell lines Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription (RT) was performed as previously described (Balanathan *et al*, 2004). β_2-microglobulin ($\beta_2 mg$) was used as a housekeeping gene for block PCR. Primer sequences were: INHA, forward: CCTGTTCTTGGATGCCTTG; reverse: AGCTGGGCTGAA GTCACCT and β_2 mg, forward: CCGTGTGAACCATGTGACTT; reverse: CAAACATGGAGACAGCACTC. Absolute quantitative real-time analysis was used to assess the levels of TGFβRIII mRNA expression in the clones. The analysis was performed on a LightCycler real-time PCR machine (Roche Diagnostic, Mannheim, Germany) using LightCycler Fast Start DNA Master SYBR Green 1 (Roche Diagnostic) according to the manufacturer's instructions. All experiments were carried out twice and duplicate readings were taken for each replicate. The quantity of mRNA was determined using a standard curve and all values were normalised using the housekeeping gene, hypoxanthine ribosyl transferase (*HPRT*). Primer sequences were: TGFβRIII, forward: TTCCCTGTTCACCC GACCTGAAAT; reverse: CGTCAGGAGGCACACATTA and HPRT, forward: TGTAATGACCAGTCAACAGGG; reverse: TGGCTTATAT CCAACACTTCG.

Confirmation of protein expression by ELISA Cell lysates and conditioned media (conditioned for 24 h) were prepared from EV- and INHα-transfected clones. Total protein (1 μg μl^{-1}) was used for further analysis. Inhibin A and B and activin A concentrations were measured in triplicate using specific ELISA according to the manufacturer's instructions (Diagnostic Systems Laboratories, Webster, TX, USA). VEGF-A and VEGF-C ELISAs were measured in duplicate using specific ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Two biological replicates were examined.

Direct cell counting: proliferation assay LNCaP and PC3 cells were seeded at a density of 1×10^5 cells per well and 5×10^3 cells per well, respectively, in 24-well plates and incubated at 37°C. Triplicate wells were harvested by trypsinisation on days 1, 2, 3, 4 and 5, and numbers of cells per well were counted using haemocytometer. Each experiment was repeated twice. The results obtained from individual clones (EV and INHα) were pooled for each treatment.

Scratch wound assay: motility assay Cells were plated in triplicate in 6- or 12-well plates and grown until approximately 70–80% confluence. The cell monolayer was then wounded and analysed over time as previously described (Sharp *et al*, 2004). Each experiment was repeated twice. The results obtained from individual clones (EV and INHα) were pooled for each treatment.

Intra-prostatic inoculation of LNCaP and PC3 cells The experiments were in accordance with NHMRC of Australia guidelines. LNCaP (2×10^6) or PC3 (5×10^5) clones were injected orthotopically into the ventral lobe of the prostate gland (10 animals per clone) of male SCID mice as previously described (Zeng *et al*, 2006). After 7–9 weeks, mice were killed and primary prostate tumours were removed and weighed. In addition, regional lymph nodes were removed for analysis. Monoclonal human mitochondria antibody (1 : 100; Chemicon, Temecula, CA, USA) was used to

determine the presence of human cells in the tumours as previously described (McCulloch *et al*, 2005). The monoclonal R1 antibody (7.5 $\mu g\,ml^{-1}$), kindly provided by Dr Nigel Groome, was used to determine INHα expression in tumours as previously described (Balanathan *et al*, 2004).

Lymph node volumes were determined using stereological analysis as previously described (McPherson *et al*, 2001). The lymph nodes were serially sectioned at 5 μm thickness and using a random sampling scheme, every 20th section was chosen for analysis. Briefly, the computer program newCAST component (version 2.14; Visiopharm, Hørsholm, Denmark) was used to generate a point grid, and volumes of the lymph nodes were determined. Each section was examined using $\times 20$ magnification and tissue sections were mapped to define tissue boundaries and were sampled at predetermined intervals along *x*- and *y*-axes using a single grid-counting frame. The volume was then determined using the equation = no of points for each tissue $\times$ area per point $\times$ distance; in this case the distance was defined by thickness of the sections (5 μm) plus (5 μm $\times$ 20 for every 20th section).

LVD in the intra-prostatic tumours Lymphatic vessels were identified using lymphatic vascular endothelial hyaluronan receptor (LYVE-1), a marker of lymphatic endothelium (Banerji *et al*, 1999). Invasion of tumour cells into lymphatics was monitored by the presence of human mitochondrial protein-stained cancer cells in lymph vessels. Double immunostaining for LYVE-1 and mitochondria was performed on a Dako Autostainer (Dako, Glostrup, Denmark). The sections were incubated with LYVE-1 antibody (Fitzgerald, Boston, MA, USA) diluted at 0.5 $\mu g\,ml^{-1}$ for 2 h. LYVE-1 was detected by incubation with Envision polymer-anti-rabbit-HRP (Dako) for 15 min and visualised with diaminobenzidine (Dako). Sections were then incubated with Double Staining Enhancer (Zymed, San Francisco, CA, USA) for 15 min and exposed to mitochondrial antibody (Chemicon) diluted at 1 : 200 for 2 h. Secondary antibody, biotinylated rabbit anti-mouse IgG1 (Zymed) was applied and the immunoreactivity was detected by ExtrAvidin-Alkaline phosphatase (Sigma, St Louis, MO, USA) and visualised by reaction with Vector Red (Vector Laboratories, Burlingame, CA, USA). The sections were counterstained with hematoxylin (Dako) and immunolocalisation was examined using an Olympus BX-60 microscope (Tokyo, Japan).

Lymphatic vessels were counted using stereological methods as previously described (Balanathan *et al*, 2004). Lymphatic vessels were counted within tissue sections (of randomly selected INHA-positive prostate tumours, $n = 15$ and EV tumours, $n = 11$; using $n = 2$ randomly selected sections per tumour) to assess the LVD within the tumour (intra-tumoural) region, the region in contact with both the tumour and the stroma (peritumoural) and the region away from tumour. LVD was expressed as the number of lymph vessels per mm^2.

Statistical analyses All statistical analyses were performed and results were analysed by ANOVA or *t*-tests as specified. The relationships between INHα expression and clinicopathological parameters were evaluated by Fisher's exact test. The mean staining intensity of patients positive for each of the respective clinicopathological parameter was compared to the mean staining intensity (reference) of those patients who were negative. The relative risks and 95% confidence intervals (CI) were estimated.

RESULTS

Relationship between INHα expression and clinicopathological parameters in primary prostate adenocarcinomas

Immunostaining revealed differential expression of INHα in benign epithelial, G3/G4 cancer regions as well as in the stroma of primary PCa tissues from patients with organ-confined disease (Figure 1A–D) and those with metastasis to the lymph nodes (Figure 1E–H). Association between clinicopathological prognostic factors and INHα expression is shown in Table 1. Elevated expression of INHα in the benign epithelial regions of the primary PCa tissues showed a higher relative risk in PCa patients positive for extracapsular spread ($P = 0.01$). Similarly, elevated expression of INHα in the stroma of the primary PCa tissues showed a higher risk of PCa patients positive for extracapsular spread ($P = 0.0011$), surgical margins ($P = 0.0006$), VEGFR-3 expression ($P = 0.00067$) and lymph node metastasis ($P < 0.0001$). Further analysis showed that there was a significant increase in INHα staining in benign epithelial ($P = 0.018$) and stromal ($P < 0.0001$) regions but not in G3/G4 cancer regions in tissues from patients with lymph node metastasis compared to patients with organ-confined disease (data not shown).

Isolation and characterisation of cells over-expressing INHα

The INHα expression profile in clinical specimens suggests pro-tumourigenic and pro-metastatic function for INHα in advanced

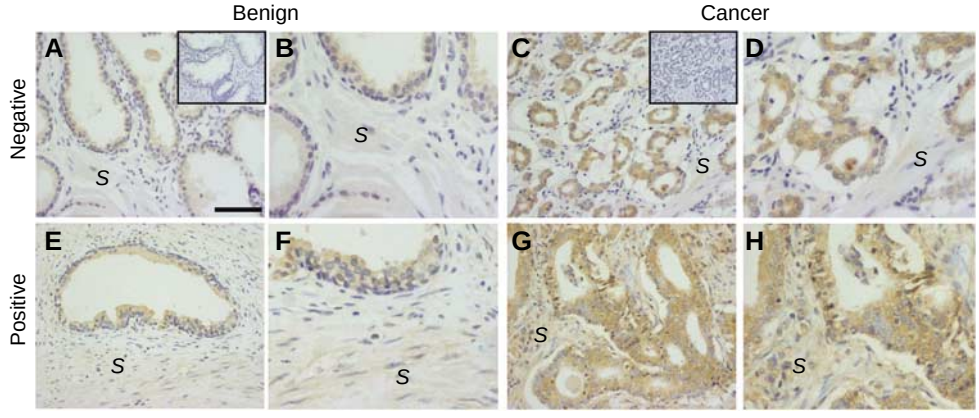

Figure 1 INHα expression in clinical specimens and its association to prostate disease. Immunohistochemical staining of INHα in primary prostate tumours from PCa patients with (**A**–**D**) organ-confined (negative) and (**E**–**H**) metastatic disease (positive). INHα immunostaining intensity in benign epithelial, cancer region (G3/G4) and stromal regions (*S*) is shown. Inset shows IgG control. Bar = 200 μm. **B**, **D**, **F** and **H** are enlargements of **A**, **C**, **E** and **G**, respectively.

Table 1 Relationships between the expression of INHα and clinicopathological parameters in prostate adenocarcinoma ($n = 37$)

		Benign regions			Cancer regions (G3/G4)			Stromal regions		
Parameters	**No. of specimens**	**Mean intensity**	**Relative risk (95% CI)**	***P*-value**	**Mean intensity**	**Relative risk (95% CI)**	***P*-value**	**Mean intensity**	**Relative risk (95% CI)**	***P*-value**
Combined Gleason grade										
6	16	0.7	NA		1.88	NA		0.30	NA	
7	14	1.51	NA		2.21	NA		1.21	NA	
⩾8	7	1.29	NA		1.99	NA		2.00	NA	
Extracapsular spread										
Positive	24	1.43			2.13			1.41		
Negative	13	0.65 (reference)	2.07 (1.04–4.13)	0.01[a]	1.87 (reference)	1.27 (0.76–2.11)	NS[a]	0.08 (reference)	2.55 (1.39–4.65)	0.0011[a]
Surgical margins										
Positive	16	1.15			1.84			1.47		
Negative	21	1.12 (reference)	1.15 (0.68–3.08)	NS[a]	2.18 (reference)	0.71 (0.32–1.53)	NS[a]	0.50 (reference)	4.75 (1.62–13.93)	0.0006[a]
VEGFR-3+ vessels										
Positive	18	1.29			2.07			1.53		
Negative	19	0.97 (reference)	1.27 (0.62–2.60)	NS[a]	2.00 (reference)	1.20 (0.0–2.39)	NS[a]	0.33 (reference)	2.85 (1.28–6.37)	0.0067[a]
Lymph node metastasis										
Positive	16	1.41			2.06			1.93		
Negative	21	1.01 (reference)	1.62 (0.67–3.97)	NS[a]	2.00 (reference)	0.84 (0.48–1.46)	NS[a]	0.15 (reference)	13.22 (1.94–90.00)	<0.0001[a]

Abbreviations: VEGFR = vascular endothelial growth factor receptor; NA = not applicable; NS = not significant; CI = confidence interval. [a]Fisher's exact test.

Table 2 Inhibin A, inhibin B and activin A protein expression in INHα clones

	Cell lysates			Conditioned media		
Cell lines	**Inhibin A ($pg\,ml^{-1}$)**	**Inhibin B ($pg\,ml^{-1}$)**	**Activin A ($ng\,ml^{-1}$)**	**Inhibin A ($pg\,ml^{-1}$)**	**Inhibin B ($pg\,ml^{-1}$)**	**Activin A ($ng\,ml^{-1}$)**
LNCaP						
L16[a]	<10	166.3	<0.011	<10	<10	<0.011
L17[a]	<10	137.4	<0.011	<10	<10	<0.011
L18[a]	<10	118.5	<0.011	<10	<10	<0.011
L1[b]	<10	433.6	<0.011	<10	88	<0.011
L5[b]	<10	183.8	<0.011	<10	24.7	<0.011
L8[b]	<10	308.7	<0.011	<10	161.8	<0.011
PC3						
P128[a]	50.1	67.2	1.960	76	<10	8.860
P129[a]	56	64.1	1.780	80.8	<10	7.520
P130[a]	48.1	73	1.720	74.6	<10	8.820
P20[b]	228.1	149.7	1.280	197	185	7.320
P103[b]	194.1	186	1.690	132.3	226.4	9.340
P104[b]	221.6	187.8	1.670	194.9	177.3	11.320

[a]EV-transfected clones. [b]INHα over-expressing clones.

PCa. Thus, to elucidate the effect of elevated levels of INHα expression on PCa cells, we stably transfected LNCaP and PC3 cell lines with an expression vector containing an INHα cDNA or with a control EV and confirmed the expression of the transgene by PCR (Supplementary Figure 1). Expression of dimeric proteins (inhibin A, inhibin B and activin A) was examined by ELISA. Analysis of cell lysates and conditioned media identified an increase in expression of inhibin B, but not inhibin A in INHα-transfected LNCaP cells and both inhibin A and inhibin B in the INHα-transfected PC3 cell lines (Table 2). There was no change in activin A level in the transfected cells. The expression of INHα protein was also validated by western blot and immunohistochemistry (data not shown). Immunohistochemistry of antibiotic selected clones revealed that the selected INHα-transfected clones were a heterogeneous population of INHα-positive and -negative cells. Because primary PCa cells are known for their heterogeneity and the aim of this study was to determine the effect of elevated levels of INHα in cancer, it was concluded the heterogeneous population was not going to bias the outcomes of the functional assays.

Growth characteristics of INHα-transfected LNCaP and PC3 clones *in vitro*

To evaluate the effect of over-expression of INHα on the growth of LNCaP and PC3 cell lines, we examined the proliferative capacity and motility of the various clones. Direct cell count demonstrated reduced proliferation of INHα over-expressing LNCaP cells (Figure 2A) and increased proliferation of PC3 cells (Figure 2B) when compared to their respective EV clones in the later stages of the growth curve. Monolayer wound healing assays showed INHα over-expressing LNCaP cells had a reduced rate of wound closure at earlier time points (Figure 2C) whereas INHα over-expressing PC3 cells demonstrated a trend towards increased rate of wound closure (Figure 2D). Because the significant changes in proliferation and motility observed in this study occur at different time points, it suggests that they are independent from each other.

Effect of INHα over-expression on tumour growth and metastasis

The influence of INHα on tumour growth and metastasis was determined *in vivo*. The clones were injected at the orthotopic site (prostate) and after 7–9 weeks primary prostate tumours as well as the adjacent lymph nodes were harvested. Primary prostate tumour size was determined and the incidence of lymph node metastasis was scored. Positive immunostaining for human mitochondrial protein confirmed that the primary and secondary tumours originated from intra-prostatic injection of human cells. INHα immunostaining was used to confirm INHα expression in tumours (Figure 3A–D, left). Immunostaining in the INHα tumours showed that INHα expression was not uniform within the tumour, which is consistent with a heterogeneous population of INHα-positive and -negative cells in the INHα-transfected clones

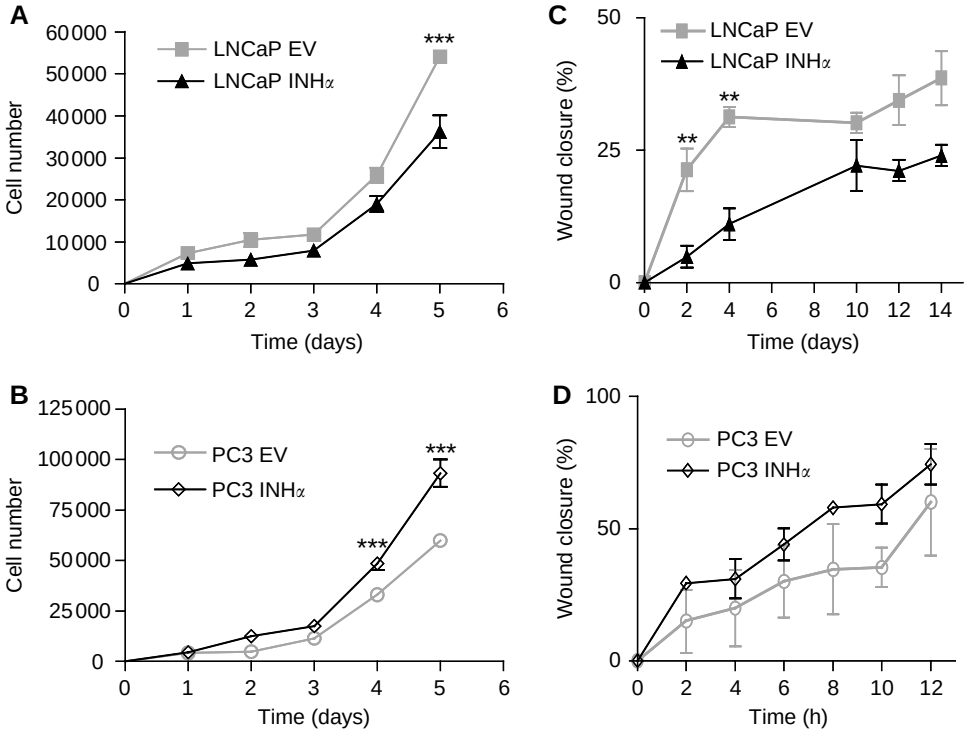

Figure 2 Effect of INHα over-expression on proliferation and motility of LNCaP and PC3 cells, *in vitro*. (**A** and **B**) Direct cell counts for EV- and INHα -transfected clones are shown; (**C** and **D**) the percentages of wound closure from EV- and INHα -transfected clones are shown. $^{**}P<0.01$, $^{***}P<0.001$ significant difference between the EV- and INHα -transfected clones. All results are from representative experiments. Data shown are mean ± s.e. of the mean.

we had observed previously. INHα immunostaining of the lymph nodes showed INHα-positive LNCaP cells to be distributed throughout the lymph node tumours whereas INHα-positive PC3 cells were confined to the outer tumour regions (Figure 3C and D, left). INHα over-expression in LNCaP cells did not affect orthotopic tumour take but significantly reduced the size of the primary tumour ($P=0.0029$) compared to the EV-transfected clones (Figure 3A, middle and right). Furthermore, INHα over-expression in LNCaP cells did not change the incidence of lymph node metastasis or the size of the lymph node tumours (Figure 3C, middle and right). INHα over-expression in PC3 cells had no effect on orthotopic tumour take but a significant increase in the primary prostate tumour size ($P=0.005$) was observed (Figure 3B, middle and right). INHα over-expression in PC3 significantly increased the incidence of lymph node tumours ($P=0.0341$) and lymph node tumour size ($P=0.0047$) compared to the EV-transfected clones (Figure 3D, middle and right). The altered tumour size following INHα over-expression may have influenced the formation of metastasis.

LVD and invasion of tumour cells into lymphatics in INHα over-expressing primary prostate tumours

Changes to LVD and lymphangiogenesis are often associated with metastatic spread of cancer cells to the lymph nodes (Mattila *et al*, 2002; Zeng *et al*, 2005). To understand the mechanisms and to provide proof of metastatic spread observed in the mice injected with INHα-positive cells, we stained LNCaP and PC3 INHα and EV orthotopic tumours for LYVE-1, and human mitochondrial antibody to determine LVD and the degree of invasion of tumour cells into lymphatic vessels (lymphatic invasion) in the tissues. Consistent with our previous study (Zeng *et al*, 2006), the analysis of LNCaP tumours in the present study was complicated by the significantly larger size of the tumours compared to PC3 tumours. This larger size resulted in numerous necrotic areas in the LNCaP tumours, making it difficult to consistently define the different tumour peripheries and the intra- and inter-tumoural regions. For this reason stereological analysis of the LNCaP tumours was not possible. However, histological evaluation of the LNCaP tumours demonstrated no LYVE-1-positive lymphatic vessels within the tumour with lymphatic vessels only present in the regions away from the tumour (Figure 4A and B). In contrast, PC3 tumours had lymphatic vessels distributed throughout the tumour (Figure 4C and D). Stereological analysis of the PC3 tumours revealed a significant increase ($P=0.0023$) in total LVD in the intra-tumoural regions with no difference in LVD in peritumoural regions and regions away from tumour in INHα-positive tumours compared to the controls (Figure 4E). A significant increase in lymphatic invasion in the intra-tumoural ($P=0.0002$), peritumoural ($P=0.0225$) and regions away from tumour ($P=0.0077$) in INHα-positive tumour tissues compared to the controls was evident (Figure 4F).

Factors inducing tumour growth and metastasis in INHα over-expressing PC3 tumours

The observed increase in LVD in INHα-positive PC3 tumours suggests that the metastatic spread of the cancer cells from the primary tumour site to the lymph nodes occurs through the process of lymphangiogenesis. Because VEGF-A and VEGF-C have been implicated in inducing/promoting metastasis to the lymph nodes in PCa (Karkkainen *et al*, 2002; Weis and Cheresh, 2005; Zeng *et al*, 2006), we went on to determine the expression of VEGF-A and VEGF-C proteins by ELISA (Table 3) in INHα- and EV-transfected clones *in vitro*. There was no significant change in the amount of VEGF-A and VEGF-C secreted by the different LNCaP clones. VEGF-C protein was significantly increased ($P=0.0011$) in the INHα over-expressing PC3 clones compared

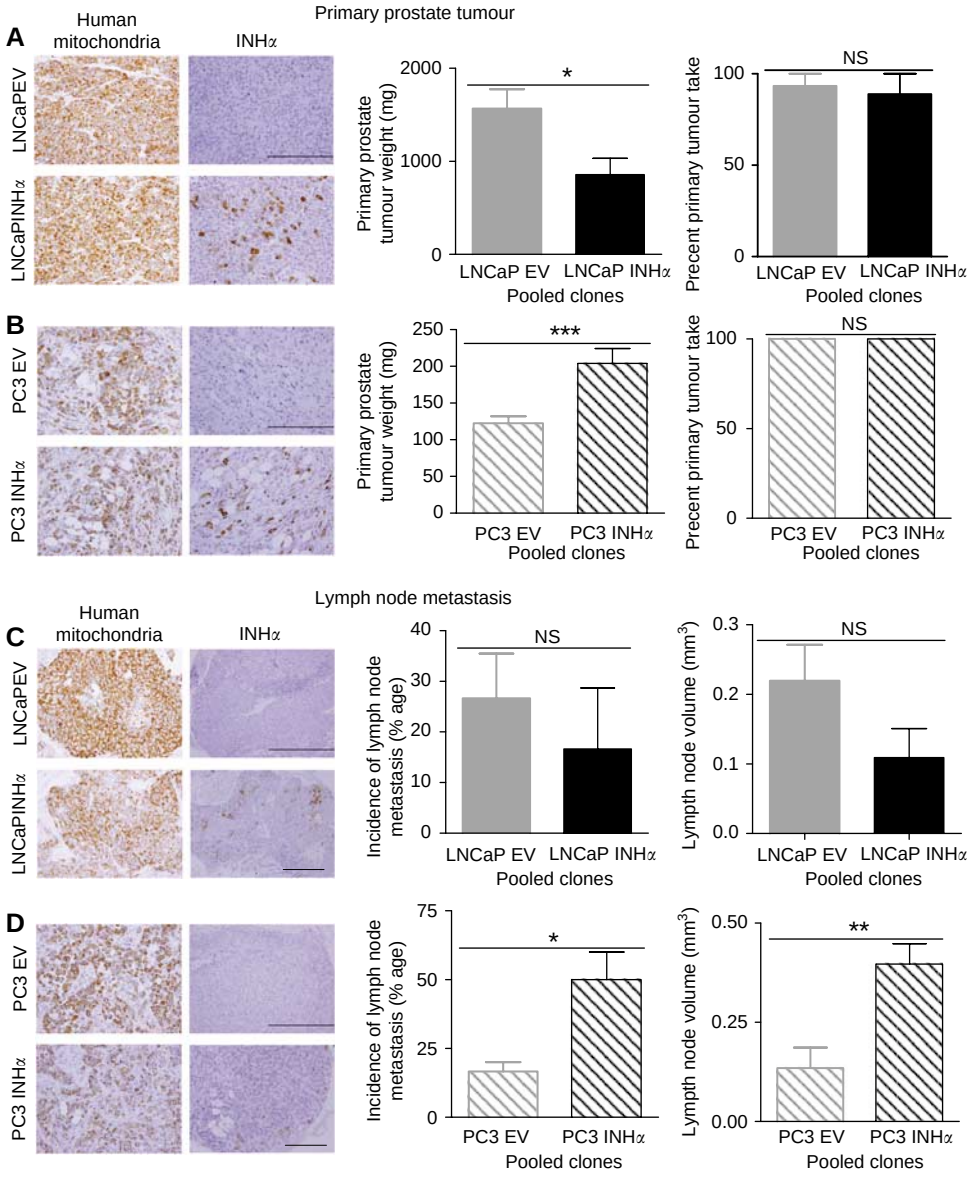

Figure 3 Effect of INHα over-expression on primary prostate tumour growth and lymph node metastasis. (**A**–**D**) Left, immunohistochemistry of primary prostate and lymph node tumours using human mitochondria and INHα staining confirmed the human origin of the cells in LNCaP- and PC3-inoculated mice and INHα expression in the tumours. Bar = 200 and 500 μm. (**A** and **B**) Primary prostate tumour weights (middle) and primary prostate tumour take (right). (**C** and **D**) Incidence of lymph node metastasis (middle) and lymph node volume (right). *$P<0.05$, **$P<0.01$, ***$P<0.001$ and no significant (NS) difference between the mean of the EV clones and the mean to the INHα-transfected clones. The bars represent EV-transfected LNCaP and PC3 clones in grey, INHα-transfected LNCaP and PC3 clones in black. Data are shown as mean ± s.e. of the mean.

to their EV clones, however there was no significant change in secreted VEGF-A levels.

Expression of TGFβRIII in LNCaP and PC3 transfected clones

Although being a receptor for TGFβ, TGFβRIII is also a receptor for inhibins (Wiater and Vale, 2003), and recent studies have reported loss of TGFβRIII as a common and important event in human PCa (Turley *et al*, 2007; Sharifi *et al*, 2007a). Down-regulation of TGFβRIII in PCa has been suggested to reflect loss of sensitivity to tumour suppressive inhibin by the PCa cells. To test this hypothesis, we determined the levels of TGFβRIII mRNA expression in the clones. Consistent with a recent study (Sharifi *et al*, 2007a), real-time analysis of PCa cell lines revealed LNCaP cells to have more TGFβRIII mRNA expression compared to PC3 cells. There was no change in TGFβRIII expression in INHα-transfected LNCaP or PC3 clones compared to EV-transfected clones (Figure 5).

DISCUSSION

Although inhibin is used as a biomarker for ovarian cancer, the biological function of elevated levels of INHα expression in PCa and certain types of ovarian cancer is unclear. This study is the first functional study to link up-regulation of INHα expression in androgen-independent prostate disease and progression of primary prostate and secondary tumours and metastasis.

We conducted a cross-sectional study to determine a link between INHα expression and a number of clinicopathological parameters: Gleason score, surgical margin status, extracapsular

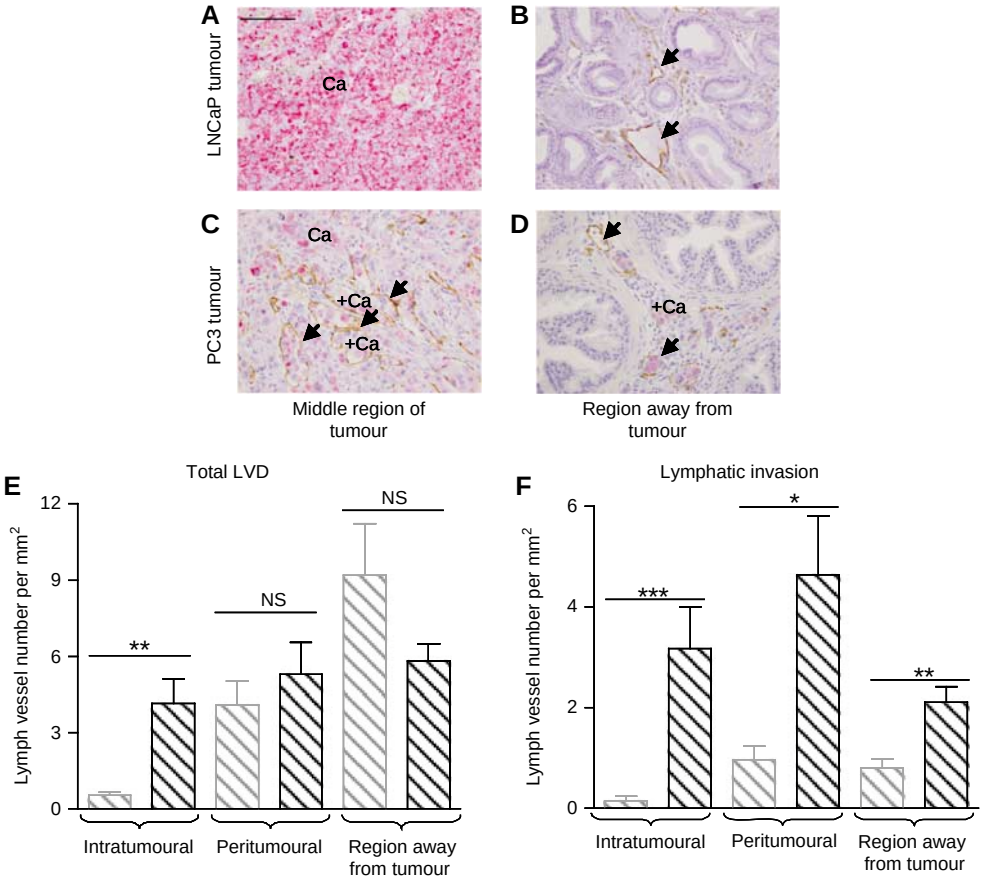

Figure 4 Effect of INHα on lymphatic vessel density and invasion. (**A**–**D**) Lymphatic vessels (LVs) were stained with LYVE-1 antibody (brown, ←) and human prostate cells (Ca) with human mitochondria antibody (purple). Bar = 200 μm. The total number of LVs (**E**) and LVs with cancer cells in their lumen (**F**) (for example, of such a vessel see '+Ca' in panels **C** and **D**) in the intra-tumoural, peritumoural and region away from tumour of the primary prostate tumour were counted. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and no significant (NS) difference between LVD in INHα over-expressing primary tumour compared to EV tumours. The bars represent EV-transfected PC3 clones in grey, INHα-transfected PC3 clones in black. Data are shown as mean ± s.e. of the mean.

Table 3 Effect of over-expressing INHα on VEGF-A and VEGF-C expression

	LNCaP		**PC3**	
	EV clones	**INHα clones**	**EV clones**	**INHα clones**
VEGF-A Secreted protein (pg ml^{-1})	1751 ± 33.48	1747 ± 26.02	1794 ± 40.31	1712 ± 34.64
VEGF-C Secreted protein (pg ml^{-1})	5.86 ± 2.66	7.82 ± 2.13	3377 ± 566.0	6892 ± 531.6**

Abbreviations: EV = empty vector; VEGF = vascular endothelial growth factor. Data from individual clones were pooled and presented above. $^{**}P = 0.0011$.

spread, lymph node status and VEGFR-3 expression, which are well-established prognostic factors of PCa (Wheeler *et al*, 1998; Cheng *et al*, 1999, 2001, 2005; Li *et al*, 2004). This study showed that an elevated level of INHα expression in the primary prostate tumour can be used as a predictive factor for prognosis in PCa. Univariate analysis showed a significant association between elevated levels of INHα in primary PCa tissues and extracapsular spread, surgical margins, VEGFR-3 expression and lymph node status. This finding is supported by our previous study that reported elevated levels of INHα in PCa patients to be associated with a higher risk of recurrence, although this association was not statistically significant (Risbridger *et al*, 2004b). The proposed cellular site for INHα expression and action is epithelial cells, however our immunostaining and analysis of the primary prostate tumour showed a significant association of elevated levels of INHα in both benign epithelial cells and stromal cells to extracapsular spread. There is increasing evidence that the surrounding microenvironment also has a major function in cancer cell growth, survival, invasion and metastatic progression, further supporting the pro-tumourigenic and pro-metastatic function of INHα in advanced PCa (reviewed in Chung *et al*, 2005; Alberti, 2006; Taylor and Risbridger, 2008). Likewise, Wheeler *et al* (1998) reported that invasion into or through the capsule of the prostate is strongly associated with both the ability of PCa to metastasise and with recurrence of cancer after radical prostatectomy. Our data link INHα expression in both epithelial and stromal cells with extracapsular spread, which suggests a role for INHα in advanced PCa. In addition, the observed up-regulation of INHα in benign epithelium and stromal regions in the primary prostate tumours in patients with lymph node metastasis suggests an association with VEGF-C-linked metastasis. Further analysis of our clinical data showed that INHα expression is stronger in benign epithelium and stroma but not the G3/G4 regions of the prostate in patients with lymph node metastasis compared to those with organ-confined

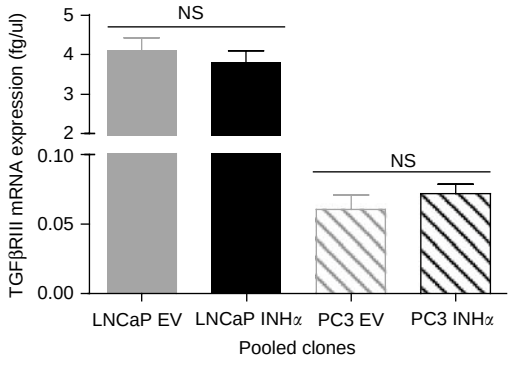

Figure 5 TGFβRIII expression in LNCaP and PC3 clones. Total RNA (2.5 μg) was reverse transcribed (RT) and absolute quantitative real-time analysis was performed to determine the levels of TGFβRIII mRNA expression in the EV- and INHα-transfected clones. The bars represent EV-transfected LNCaP and PC3 clones in grey, INHα-transfected LNCaP and PC3 clones in black. Data are shown as mean ± s.e. of the mean.

disease. In PCa, Tsurusaki *et al* (1999) found that VEGF-C mRNA levels were significantly higher in lymph-node-positive tumours and that VEGFR-3-positive vessels were increased in the stroma of VEGF-C-positive tumours. Also, VEGF-C has been shown to promote growth of lymphatic vessels into and around tumours in animal models and this was associated with metastatic spread to the lymph nodes and sometimes to distant organs (Mandriota *et al*, 2001; Skobe *et al*, 2001; Mattila *et al*, 2002). Collectively, our analysis of INHα expression in primary prostate tumour tissues and the association to well-established prognostic factors strongly supports a pro-tumourigenic and pro-metastatic function for INHα in PCa.

Because the proposed cellular site for pro-tumourigenic and pro-metastatic action of INHα is cancer epithelial cells, we over-expressed INHA in metastatic epithelial PCa cell lines, LNCaP and PC3, to evaluate functionally the effect of elevated levels of INHα in PCa. A key event in the progression of PCa is the transition from an androgen-dependent to -independent stage, where subpopulations of tumour cells either gain resistance to, or adapt to, an androgen-depleted environment, and begin to proliferate resulting in the progression to highly aggressive and metastatic androgen-independent disease. Furthermore, when PCa is advanced or metastatic it is usually incurable and tends to metastasise to bone and lymph nodes. LNCaP cells, originally isolated from a lymph node metastasis (Horoszewicz *et al*, 1980, 1983), have retained their ability to respond to androgens, whereas PC3 cells, originally derived from a bone metastasis (Kaighn *et al*, 1979), are androgen independent. Thus these cell lines represent early and late stages of metastatic prostate disease.

INHα over-expression in LNCaP cells demonstrated reduced cell proliferation, migration and primary prostate tumour growth whereas INHα over-expression in these cells did not change the cells' ability to metastasise to the lymph nodes nor did it influence the growth of the lymph node tumours. The lack of change in VEGF-C expression in INHα-transfected LNCaP cells is consistent with the unchanged metastatic ability of LNCaP cells *in vivo*. In contrast, INHα over-expression PC3 cells demonstrated increased cell proliferation, migration, primary prostate tumour and lymph node tumour growth. These cells showed increased metastasis to the lymph node, which was accompanied by an elevation of LVD and tumour cell invasion into lymphatics. These effects were associated with up-regulation of VEGF-C. Similarly, Karpanen *et al* (2001) reported that VEGF-C expression in a mouse tumour model strongly promoted the growth of tumour-associated lymphatic vessels, which in the tumour periphery were commonly infiltrated with the tumour cells. Although limited to the use of only two cell lines, the current study is the first to functionally show a pro-tumourigenic and pro-metastatic function for INHα in an androgen-independent model of metastatic prostate disease.

Another explanation for the different effects of INHα observed in the current study is the loss or gain of a yet-to-be-identified signalling pathway for INHα in either tumour suppression or promotion. We have shown that LNCaP cells express more TGFβRIII mRNA expression compared to PC3 cells and that the level of TGFβRIII mRNA expression is maintained after INHα over-expression. Loss of TGFβRIII during PCa progression has been suggested to be a reason for loss of sensitivity to the tumour suppressive effect of inhibin in prostate disease (Turley *et al*, 2007; Sharifi *et al*, 2007a, b). Whether androgen status, different cell types and/or levels of TGFβRIII expression are responsible for the different effects of inhibin observed in the present study is an important area of future investigation.

Inhibins are known to be involved with regulating members of the TGFβ superfamily, including TGFβ itself, as well as activins and BMP, by competing for their receptors or co-receptors (Wiater and Vale, 2003; Farnworth *et al*, 2006, 2007). However, unlike other members of the TGFβ superfamily (Pangas and Woodruff, 2000; Shi and Massagué, 2003), the signalling pathway(s) for inhibin have not yet been defined. Although it remains unclear how INHα mediates downstream cellular events leading to prostate disease, the observed effect of INHα in the present study suggests and supports an effect of inhibin which is independent of TGFβ/activin in PCa. Owing to the presence of both βA and βB subunits in LNCaP and PC3 cells, the clones were able to produce dimeric inhibin A and inhibin B. The presence of inhibin B in both LNCaP and PC3 cells is significant as inhibin B is the pre-dominant form of inhibin in men and therefore more relevant to the study of PCa. Although the presence of inhibin A in PC3 may be of relevance to the observed phenotypes of PC3 cells, further investigation into the functional difference resulting from inhibin A *vs* inhibin B production in these cells was beyond the scope of this project. The level of inhibin A and inhibin B produced by the cells reflects the level of the β-subunits expressed and secreted by the respective cell lines (McPherson *et al*, 1999). Our study showed no change in the levels of activin A (βAβA), which indicates that observed effects are specific to inhibin and not a response to activin levels. Therefore, although TGFβ/activins have been shown to induce expression of angiogenic/lymphangiogenic factors through P13K and/or SMAD2 pathways (Wagner *et al*, 2004; Kang, 2006) and TGFβ/activins inhibit growth of PCa cells (Wilding *et al*, 1989; Wang *et al*, 1996; McPherson *et al*, 1997), the different responses of the PCa cell lines to elevated levels of INHα we have observed cannot be simply explained by abrogation of TGFβ/activin signalling. Thus, the effect of inhibin on LNCaP cells observed in this study supports a role for inhibin in PCa independent of TGFβ/activin. Also, the increased metastatic ability of INHα observed in INHα over-expressing PC3 cells cannot be due to antagonism of activin action because of the simultaneous expression of follistatin in these cells (McPherson *et al*, 1999). Follistatin is an activin-binding protein that binds to activin with a high affinity thereby inhibiting activin bioactivity (Phillips and de Kretser, 1998). Taken together, the current study demonstrates a direct action of INHα in PCa that is context and/or cell-type dependent.

In summary, this study showed both benign epithelial and stromal regions of primary prostate tumours to be the sites of INHα expression. We have also demonstrated a strong association of elevated levels of INHα to well-established prognostic factors of PCa. This finding has identified INHα as a very important predictive factor that can be used to identify patients at increased risk for disease progression and cancer death after radical prostatectomy, so that appropriate therapy can be selected. Finally, the current

study has shown a pro-tumourigenic and pro-metastatic role for INHα in an androgen-independent model of metastatic prostate disease. This suggests that in the absence of androgens, elevated levels of inhibin may be driving the more aggressive and metastatic phenotypes of PCa tumour.

ACKNOWLEDGEMENTS

We thank Dr Christopher Bulter for critically reviewing this paper and Dr Stephen McPherson for his assistance with stereological analysis. Post-doctoral training award by United States Department of Defense (PB), Cancer Council Victoria grant-in-aid (EDW), Cancer Institute of New South Wales (LGH), Senior Research Fellowship from Australian National Health and Medical Research Council (NHMRC) and the Pfizer Foundation (MGA and SAS) and by an NHMRC program grant. Principal Research Fellowship from Australian NHMRC (GPR).

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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APPENDIX 2: EPWORTH HREC APPROVAL

Epworth Hospital
ABN 97 420 694 950
Epworth Foundation
89 Bridge Road Richmond Vic 3121 Australia T: 61 3 9426 6666 F: 61 3 9427 0353 www.epworth.org.au
Epworth

27 December 2006

Prof. Gail Risbridger
c/o Courtney Bamford
Centre for Urological Research
Monash Institute of Medical Research
27 Wright Street,
CLAYTON VIC 3168

Dear Prof. Risbridger,

Re: ROLE OF TUMOUR STOMA IN PROSTATE CARCINOGENISIS
Epworth Study No 34306

Thank-you for your requested amendments and correspondence dated the 15th of November 2006.

The Epworth Healthcare HREC accepted these amendments at their meeting on the 6th of December 2006.

Thank-you for keeping the committee updated on the progress of your study and we look forward to receiving your annual report in due course.

Yours sincerely,

Louise Grey
HREC Coordinator
Epworth Hospital
89 Bridge Rd.
RICHMOND VIC 3121

Epworth Eastern
1 Arnold Street Box Hill Vic 3128 Australia
T: 61 3 8807 7100 F: 61 3 8807 7800

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89 Bridge Road Richmond Vic 3121 Australia
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85 Wilson Street Brighton Vic 3186 Australia
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APPENDIX 3: SCERH APPROVAL

From scerh <scerh@adm.monash.edu.au>

Sent Wednesday, June 13, 2007 12:59 pm

To Renea Taylor <Renea.Taylor@med.monash.edu.au> , Gail Risbridger <Gail.Risbridger@med.monash.edu.au> , Preetika Balanathan <Preetika.Balanathan@med.monash.edu.au>

Cc

Bcc

Subject Monash Human Ethics - 2004/145MC: Role of tumor stroma in prostate carcinogenesis - Request for Amendment

PLEASE NOTE: To ensure speedy turnaround time, this correspondence is now being sent by email only. If you would prefer a hard copy on letterhead, please contact the Human Ethics Office (9905 2076 or scerh@adm.monash.edu.au) and a hard copy will be posted to you.

We would be grateful if first-named investigators could ensure that their co-investigators are aware of the content of the correspondence.

Dr Renea Taylor
Centre for Urological Research
Faculty of Medicine, Nursing and Health Sciences
Monash Medical Centre Campus

13 June 2007

2004/145MC: Role of tumor stroma in prostate carcinogenesis

Dear Researchers,

Thank you for submitting further information as requested by the Standing Committee on Ethics in Research Involving Humans (SCERH) with respect to the Request for Amendment to the above named project.

This is to advise that the requested amendments dated 2 April 2007, received in our office on 5 April 2007, have been approved and the project can proceed according to your approval given on 22 April 2004.

Monash SCERH therefore approves your submission approved by the Epworth Human Ethics Committee, for Epworth study 34306, on 6 December 2006.

Thank you for keeping the Committee informed.

Mrs Lyn Johannessen
Acting Human Ethics Officer (on behalf of SCERH)

Cc: Prof Gail Risbridger, Dr Preetika Balanathan

APPENDIX 4: MMCA APPROVAL

MONASH INSTITUTE OF MEDICAL RESEARCH
AN INITIATIVE OF MONASH UNIVERSITY AND SOUTHERN HEALTH

Memo

To: Prof. Gail Risbridger, MIMR
From: Glenda Johnson
Date: 20 March 2007
Subject: Notice of Approval – Project No. MMCA 2006/45

Are inhibin-α and VEGF-C responsible for spread of cancer cells in our model of advanced prostate cancer?

Please find attached a copy of the Final Approved proposal.

The project is approved from 01/01/07 to 31/12/2010 subject to the following conditions.

1. An Annual Report must be provided each January.
2. A Final Report is submitted to the MMC Animals Ethics Committee within six months of completion of the project.
3. Unexpected or adverse events, which impact on the welfare of the animals, must be immediately reported to the Chairperson of the AEC.
4. Any changes to location of animal housing or experimental location details are to be forwarded immediately to the AEC.
5. Special Conditions - None
6. Special Responsibilities by Animal House Staff – None
7. Approved Animal Usage is:-

SPECIES	Total No.
Mouse SCID 6-8 week old/ male	140

Please destroy any previous versions of the proposal and replace with the enclosed authorized document.

Regards,

Glenda Johnson
MMC Animal Ethics Committee 'A'
MIMR, Level 3
MMC Clayton
Tel. 9594-7342
Email. Glenda.johnson@med.monash.edu.au

APPENDIX 5: SCERH APPROVAL

MONASH University

Standing Committee on Ethics in Research Involving Humans (SCERH)
Research Office

Prof Gail Risbridger
Department of Centre for Urological Research
Faculty of Medicine, Nursing and Health Sciences
Monash Medical Centre Campus

16 May 2007

CF07/0854 - 2007/0223HT: Protein expression patterns in human prostate cancer tissues

Dear Researchers,

Thank you for the information provided in relation to the above project. The items requiring attention have been resolved to the satisfaction of the Standing Committee on Ethics in Research Involving Humans (SCERH). Accordingly, this research project is approved to proceed.

Terms of approval

1. This project is approved for five years from the date of this letter and this approval is only valid whilst you hold a position at Monash University.
1. It is the responsibility of the Chief Investigator to ensure that all information that is pending (such as permission letters from organisations) is forwarded to SCERH, if not done already. Research cannot begin at any organisation until SCERH receives a letter of permission from that organisation. You will then receive a letter from SCERH confirming that we have received a letter from each organisation.
2. It is the responsibility of the Chief Investigator to ensure that all investigators are aware of the terms of approval and to ensure the project is conducted as approved by SCERH.
3. You should notify SCERH immediately of any serious or unexpected adverse effects on participants or unforeseen events affecting the ethical acceptability of the project.
4. The Explanatory Statement must be on Monash University letterhead and the Monash University complaints clause must contain your project number.
5. **Amendments to the approved project:** Changes to any aspect of the project require the submission of a Request for Amendment form to SCERH and must not begin without written approval from SCERH. Substantial variations may require a new application.
6. **Future correspondence:** Please quote the project number and project title above in any further correspondence.
7. **Annual reports:** Continued approval of this project is dependent on the submission of an Annual Report. Please provide the Committee with an Annual Report determined by the date of your letter of approval.
8. **Final report:** A Final Report should be provided at the conclusion of the project. SCERH should be notified if the project is discontinued before the expected date of completion.
9. **Monitoring:** Projects may be subject to an audit or any other form of monitoring by SCERH at any time.
10. **Retention and storage of data:** The Chief Investigator is responsible for the storage and retention of original data pertaining to a project for a minimum period of five years.

All forms can be accessed at our website www.monash.edu.au/research/ethics/human/index.html

We wish you well with your research.

Mrs Lyn Johannessen
Acting Human Ethics Officer (on behalf of SCERH)

Cc: Dr Preetika Balanathan, Prof Rob Sutherland, Dr Elizabeth Williams

Postal - Monash University, VIC 3800, Australia
Building 3E, Room 111, Clayton Campus, Wellington Road, Clayton
Telephone +61 3 9905 5490 Facsimile +61 3 9905 1420
Email scerh@adm.monash.edu.au www.monash.edu.au/research/ethics/human/index.html
CRICOS Provider No. 00008C ABN 12 377 614 012

APPENDIX 6: TGFβ – KEYSTONE SYMPOSIA ABSTRACT

Shift in the tumor suppressive activity of inhibin-α subunit during the transition from androgen-dependent to androgen-independent prostate cancer

Preetika Balanathan[1], Elizabeth D Williams[2], Hong Wang[1], Marc G Achen[3], Steven A Stacker[3], Gail Risbridger[1]. [1]Centre for Urological Research, [2]Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Melbourne, Victoria, Australia. [3]Ludwig Institute for Cancer Research, Post Office Box 2008, Royal Melbourne Hospital, Melbourne, Victoria, Australia.

The inhibin field has been perplexed by the information that inhibin-α subunit (INHA), a member of the TGFβ superfamily is a tumor suppressor in mice yet is elevated in women with ovarian cancer. Similarly we have observed up- and down-regulation of INHA expression in prostate cancer (PCa) dependent on the stage of disease. We proposed that INHA is tumor suppressive in androgen-dependent (AD) stage of the disease but loses its tumor suppressive activity or gains metastatic properties in androgen-independent (AI) stage of the disease. Recently, loss of TGFβ receptor RIII (TGFβRIII), a receptor for inhibin has been proposed to be an explanation for the different activities of INHA in PCa.

We evaluated the functional role of INHA in two well known PCa cell lines which differ in behavior and molecular makeup and have close resemblance to primary prostate disease. The AD, LNCaP and AI PC3 cell lines were stably transfected with cDNA for INHA and evaluated for their sensitive to INHA expression in the presence of endogenous levels of TGFβRIII. Over-expression of INHA in AD LNCaP cells decreased cell proliferation and migration and reduced tumor growth supporting the role of INHA as a tumor suppressor. In contrast, over-expression of INHA in AI PC3 cells increased cell proliferation, migration, tumor growth and metastasis. This supports the loss of tumor suppressive activity or gain in metastatic properties for INHA in AI stage of the disease. The shift in the tumor suppressive activity of INHA was further evident by increase in lymph node metastasis in the INHA over-expressing PC3 tumors which was accompanied by an elevation of lymphatic vessel density and tumor cell invasion into lymphatics. These effects were associated with up-regulation of the lymphangiogenic growth factor, VEGF-C. Consistent with other studies our work revealed that LNCaP cells expressed significantly more TGFβRIII mRNA than PC3 cells.

Our results demonstrate that tumor suppressive activity of INHA is altered during the transition from AD to AI PCa. It provides the first functional evidence which suggests that loss in the tumor suppressive activity of INHA in different stages of prostate disease may be due to loss in TGFβRIII expression.

Financial Support: These studies were supported by Australian National Health and Medical Research Council program grant and a Post-doctoral training award by United States Department of Defense [PB, Grant#: PC060112].

APPENDIX 7: LORNE CANCER ABSTRACT

Tumor suppressive activity of inhibin-α subunit is altered during the transition from androgen-dependent to androgen-independent prostate cancer

Preetika Balanathan[1], Elizabeth D Williams[2], Hong Wang[1], Marc G Achen[3], Steven A Stacker[3], Gail Risbridger[1]. [1]Centre for Urological Research, Monash Institute of Medical Research, Monash University, Melbourne, Victoria, Australia. [2]Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Melbourne, Victoria, Australia. [3]Ludwig Institute for Cancer Research, Post Office Box 2008, Royal Melbourne Hospital, Melbourne, Victoria, Australia.

The transition from androgen-dependent (AD) to androgen-independent (AI) disease is a key event in prostate cancer (PCa) progression and Inhibin-α subunit (INHA) has been proposed to have a tumor suppressive and pro-metastatic role during different stages of the disease. Recently, loss of TGFβ receptor RIII (TGFβRIII), a receptor for inhibin has been proposed to be an explanation for the different activities of INHA in PCa.

The AD, LNCaP and AI PC3 cell lines were evaluated for their sensitive to INHA expression in the presence of endogenous levels of TGFβRIII. Over-expression of INHA in AD LNCaP cells decreased cell proliferation, migration and reduced tumor growth supporting the role of INHA as a tumor suppressor. In contrast, over-expression of INHA in AI PC3 cells increased cell proliferation, migration, tumor growth and metastasis supporting the loss of tumor suppressive activity/gain in metastatic properties for INHA in AI stage of the disease. The shift in the tumor suppressive activity of INHA was further evident by increase in lymph node metastasis in the INHA over-expressing PC3 tumors which was accompanied by an elevation of lymphatic vessel density, tumor cell invasion into lymphatics and up-regulation of VEGF-C. Consistent with other studies our work revealed that LNCaP cells expressed significantly more TGFβRIII mRNA than PC3 cells. Analysis of human PCa specimens showed that INHA expression cannot be used to determine lymph node status in PCa patients. However, increase in INHA expression by normal epithelium and prostate intraepithelial neoplasia (PIN) regions of the tumors in patients with lymph node metastasis suggests that INHA may have a paracrine role that, directly or indirectly, promotes the spread of cancer cells from the primary prostate tumor to the lymph nodes.

Our results demonstrate that tumor suppressive activity of INHA is altered during the transition from AD to AI PCa. It provides the first functional evidence which suggests that loss in the tumor suppressive activity of INHA in PCa progression may be due to loss in TGFβRIII expression.

APPENDIX 8: GSK GRANT

GlaxoSmithKline

1061 Mountain Highway
Boronia Victoria 3155
PO Box 168 Boronia 3155
Australia
Tel. +61 03 9721 6000
Fax. +61 03 9729 5319
www.gsk.com.au

3 October 2007
Ref: DR07/091

Professor Gail Risbridger
Director
Centre for Urological Research
Monash Institute of Medical Research
Monash University
27-31 Wright St
Clayton VIC 3168

Marg
Rocco will need to sign this plse.
t Prue

Dear Professor Risbridger,

GSKA Post Graduate Support Grant – 2007 Application Round

On behalf of Dr Michael Elliott, Vice President & Area Medical Director, Australasia & Asia-Pacific, I am delighted to inform you that your application for the GSKA Post Graduate Support Grant has been successful. You may be interested to learn that your application was one of 9 selected for funding from a pool of 319 applications.

Please find enclosed duplicate copies of the Research Agreement for the abovementioned grant. The award will be $25,000 over two years.

For your action:

1. Arrange for both copies of the enclosed Research Agreement to be executed by authorised representatives for your institution.

2. Retain one copy of the Research Agreement for your records and return the second executed copy to myself using the enclosed self addressed envelope, **by no later than Friday 2 November 2007**.

3. Institution to raise a tax invoice for the amount of $16,500 (incl GST) being for the first payment, and forward to me **by no later Friday 2 November 2007.** (Please include Institute's banking details to enable GSK to make payments by Electronic Funds Transfer).

We would also like to publish the 2007 grant winners details on our website and possibly through a media release. No project details would be disclosed other than the project title. Could you please advise us if you would prefer that we do not publish this information.

GlaxoSmithKline Australia Pty Ltd
ABN 47 100 162 481

I would like to take this opportunity to congratulate you and Preetika Balanathan on your successful application. GSK looks forward to hearing about the progress on your exciting project.

Yours sincerely,
GlaxoSmithKline Australia Pty Ltd

Ashley Bates, PhD
Head of R&D Alliances Aust/NZ

Encl.

cc: Preetika Balanathan

APPENDIX 9: ANZ GRANT

ANZ Trustees
Philanthropy Partners
Level 4, 100 Queen Street, Melbourne Vic 3000
GPO Box 389D, Melbourne Vic 3001
Telephone 03 9273 6799
Facsimile 03 9273 6354

15 October 2007

PROCESSED

Dr Preetika Balanathan
Monash University
Monash Institute of Medical Research
27-31 Wright Street
Clayton Vic 3168

Dear Dr Balanathan

Project name:	**Dr Preetika Balanathan, Professor Gail Risbridger - Dual or multi-functionality of inhibin-a subunit in prostate cancer progression**
Organisation:	**Monash University**
Application date:	**29 June 2007**
Reference number:	**CT 9012**

On behalf of the Trustees of **Medical Research & Technology in Victoria - The William Buckland Foundation**, I am pleased to advise that your organisation has been granted an amount of **$15,000** to be used towards the project described in your application.

With this letter we have included:
- Grant conditions
- Advice on acknowledging The William Buckland Foundation
- Reporting format

The grant is to be expended in Victoria only. Banking the enclosed cheque indicates acceptance of the grant for the purpose of the project outlined in your application and the grant conditions attached to this letter.

As set out in condition 7, your organisation is required to submit annual progress reports and a final report (or only a final report in the case of a one year project) on the project.

The Trustees wish you every success with the project and look forward to watching the progress of your project.

Yours sincerely

Trisha Broadbridge
Manager, Philanthropy Partners, ANZ Trustees Limited

Enc

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APPENDIX 10: TRAVEL SCHOLARSHIP

KEYSTONE SYMPOSIA

Connecting the Scientific Community

28 January, 2008

Preetika Balanathan
Centre for Urological Research
Monash Institute of Medical Research, Monash University
27-31 Wright Street
Melbourne, Victoria 3168 Australia

Dear Preetika Balanathan:

Congratulations on winning a scholarship for up to $1000.00 for reimbursement of costs associated with travel to the Keystone Symposia meeting TGF-ß Family in Homeostasis and Disease to be held at Eldorado Hotel & Spa, Santa Fe, New Mexico on February 3 - 8, 2008.

Immediately upon your return home, mail your original registration, air travel, ground transportation and conference lodging receipts totaling up to $1000 to me using the pre-addressed envelope. Remember, we do not reimburse meals, incidentals or recreational expenses.

I recommend that you don't use "express check-out" upon departure from the hotel. It is very difficult to get an original receipt after you have left. It would be best to pick up your itemized receipt before leaving the hotel. If you are sharing accommodations, ask for a separate receipt with your name and portion of the hotel bill.

We can accept copies of the receipts *only* if we are reimbursing your institute, in which case, please include any identifying information; i.e. name to whom the check should be made payable, department name or account number, etc. We can also accept print outs from air travel arrangements made on the internet if they include your name, departure, destination, dates and the cost. All receipts submitted should include this information. Other allowable expenses are meeting registration, ground transportation from the airport to the meeting, car rental or mileage if using a personal vehicle. We will not reimburse credit card receipts or statements so be sure to obtain all original receipts at time service is rendered. If a wire transfer is requested a $20.00 USD fee will be deducted from the award. The cost incurred by us is $40.00 USD.

Reimbursement checks will be processed as soon as *all* receipts from *all* scholarship winners have been received. **If I do not hear from you nor receive your receipts in our office on or before 21 *February 2008* your scholarship award will be forfeited. Please do not delay in sending your receipts; it will be unfair to the others.**

When sending your receipts please go into your account on our website www.keystonesymposia.org and in "Student/Postdoc Scholarship Application" under "Award Information" note the mailing address to which you would like us to mail your reimbursement check to. You can check the status of your receipts in your account as well, e.g. "Not Received", "Received", "Processed", "Payment Sent".

Please do not call our office to check on the status of the receipts. You can check it on our website n your account in "Student/Postdoc Scholarship Application" under "Receipt Status", e.g. "Not Received", "Received", "Processed", "Payment Sent". This information gets updated regularly.

Please also note that reimbursement can be issued only in your name or in the name of your institute.

If you have any questions, you can contact me at 1-800-253-0685 ext.140 or 970-262-1230 ext.140 or at ksenias@keystonesymposia.org.

Enjoy the conference!

Ksenia Shambarger
Scholarships

Program Development & Implementation • 221 Summit Place #272 • PO Box 1630 • Silverthorne, CO 80498
800-253-0685 • 970-262-1230 • 970-262-0311 (fax) • programs@keystonesymposia.org • www.keystonesymposia.org

APPENDIX 11: ESA ANNUAL MEETING ABSTRACT 2009

Elevated level of inhibin-α subunit is pro-tumourigenic and pro-metastatic and associated with extracapsular spread in advanced prostate cancer

P. Balanathan[1], E. D. Williams[2], H. Wang[1], B. L. Eckhardt[4], J. S. Pedersen[5], L. G. Horvath[6], M. G. Achen[3], S. A. Stacker[3], R. L. Anderson[4], G. P. Risbridger[1]

[1] Centre for Urological Research, MIMR, Monash University, Clayton, VIC, Australia
[2] Centre for Cancer Research, MIMR, Monash University, Melbourne, VIC, Australia
[3] Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Melbourne, VIC, Australia
[4] Research Division, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia
[5] Tissupath Pty Ltd, Melbourne, VIC, Australia
[6] Cancer Research Program, Garvan Institute of Medical Research, Sydney, NSW, Australia

The biological role of inhibin-α subunit (INHα) in prostate cancer (PCa) is currently unclear. A recent study associated elevated levels of INHα in PCa patients with a higher risk of recurrence. This prompted us to use clinical specimens and functional studies to investigate the pro-tumourigenic and pro-metastatic role of INHα. We conducted a cross-sectional study to determine a link between INHα expression and a number of clinicopathological parameters including Gleason score, surgical margin, extracapsular spread, lymph node status and VEGF-R3 expression which are well established prognostic factors of PCa. In addition, using two human PCa cell lines (LNCaP and PC3) representing androgen-dependent and androgen-independent PCa respectively, this study investigated the biological role of elevated levels of INHα in advanced cancer. Elevated expression of INHα in primary PCa tissues showed a higher risk of PCa patients being positive for clinicopathological parameters outlined above. Over-expressing INHα in LNCaP and PC3 cells demonstrated two different and cell-type specific responses. INHα-positive LNCaP demonstrated reduced tumour growth while INHα-positive PC3 cells demonstrated increased tumour growth and metastasis via the process of lymphangiogenesis. Gene array studies suggest that the pro-metastatic effect of INHα may be due to an alteration in the ERK/MAK pathway. This study is the first to demonstrate a pro-tumourigenic and pro-metastatic role for INHα associated with androgen-independent stage of metastatic prostate disease. Our results also suggest that INHα expression in the primary prostate tumour can be used as a predictive factor for prognosis of PCa.